

**UNIVERSITY OF GHANA
COLLEGE OF HEALTH SCIENCES**

**CHARACTERIZATION AND GENOTYPING OF ROTAVIRUSES FROM
COMMUNITIES IN AKUSE DISTRICT AND ITS ENVIRONS.**

BY

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DECLARATION

I, Frederica Lizz Hayford, hereby declare that except for reference to other people's work, which I have duly cited, this thesis is the result of an original research work and that the material has not been presented either in whole or in part elsewhere for another degree and all experimental works was performed by me under the supervision of Prof. Theophilus K. Adiku.

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ABSTRACT

Background: Rotaviruses are recognised world-wide as the principal aetiological agents of severe neonatal diarrhoea in a number of species, including man and domestic animals. Eight species of this virus have been detected and classified as A, B, C, D, E, F, G and H. Human beings are predominantly infected by species A, B and C, but A–E species cause disease in other animals. Studies in Ghana have reported new emerging strains of the virus in paediatric diarrheal cases some of which have been identified as animal genotype whilst others have been identified as re-assorted variants resulting from both human and animal rotavirus strains. Investigations have proven that close co-living of humans and animals may have resulted in such interspecies infections due to recombination of the human and the animal variants of the rotaviruses, by close contact with animal faecal material. Uncommon genotypes also contribute to the rotavirus associated diarrhoeal infection and could reduce the effectiveness of the vaccines in use and hence vaccinated individuals may not benefit.

Aim: To characterize as well as determine genotypic variations in rotavirus isolates from humans and animals living in close association in communities in Akuse and its environs.

Methods: Stool samples collected from households in which humans and animals live in close association in Akuse and its environs were subjected to PAGE analysis and RNA from the stool samples were analysed by SDS-PAGE and RT PCR to determine genotypes circulating in the communities.

Results: In this study, samples were taken from 22 households in Akuse and its environs for rotavirus characterisation and genotyping. Out of the 22 households in this study, 7 (31.8%) were characterised by PAGE as positive to rotaviruses A or B, or C phenotype. In households characterised by PAGE as positives, genotype G8 was confirmed in 4

(57.1%) while both genotype G8 and G10 were confirmed in only 1 (14.3%) of the households. The amplicons for nest 1 did not genotype with the set of primers available in 3 (42.9%) of the households characterised by PAGE as positives. Among the households characterised by PAGE as positive, pherotypes A, and C were found in 6 (85.7%) and 1 (14.3%) households, respectively, while both pherotype A and B were found in 1 (14.3%) household. Majority of positives were found in humans.

Conclusion: This study has identified possible reassortant strains of rotaviruses G8 and G10 characteristically of bovine origin in humans from the Akuse district and its environs.

DEDICATION

I dedicate this piece of work to the almighty God who has showered so much of his Grace and love on me throughout the period of my Education. This work is also dedicated to my beloved daughters Stephanie, Godslove and Frances Abutiate for their immense courage to stand with me through it all. Finally, I owe this work to all my loving and caring friends who helped both in kind and with words of encouragement.

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LIST OF ABBREVIATIONS

AgNO ₃	silver nitrate
AMV	avian myeloblastosis virus
APS	ammonium persulphate
Bp	base pair
cDNA	complementary DNA
CDC	Centre for Disease Control
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DDW	double distilled water
DEPC	diethyl pyrocarbonate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dsRNA	double stranded RNA
EDTA	ethylene di-aminetetracetic acid
EIA	enzyme immunoassay
EM	Electron Microscopy
G	gram
GITC	guanidine isothiocyanate
HCL	hydrochloric acid
HRV	human rotavirus
I/II	subgroup I/II specificity

KB	kilobase
M	molar
MgCl ₂	magnesium chloride
ml	milliliter
mM	millimole
mRNA	messenger RNA
M _w	molecular weight
NaAc	sodium acetate
NaOH	sodium hydroxide
Nm	nanometre
Non I/II	non-subgroup I or II specificity
NSP	Non-Structural Proteins
PAGE	Polyacrylamide Gel Electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	hydrogen ion concentration
RNA	ribonucleic acid
RPM	revolutions per minute
RRV	rhesus reassortant rotavirus
RRV-TV	rhesus reassortant rotavirus tetravalent vaccine
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate
VP4	viral structural protein (4)
VP7	viral structural protein (7)

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND

Rotaviruses are leading aetiological agents of severe neonatal diarrhoea in distinct species (including human, especially in children) and domestic animals (e.g. calves, lambs and pigs) (Hajnalka Papp *et al.*, 2014; Kapikian and Chanock, 1990). Primarily, infection takes place in the jejunum specifically in the differentiated enterocytes of the small intestines responsible for digestion and absorption (Moon, 1994). Obliteration of the enterocytes may generate into food nutrient and water loss, causing malnutrition and dehydration that can lead to death. Subsequent infections are less severe than the previous due to immunity developed from the previous infection and adults are rarely affected (Grimwood & Lambert, 2009).

The rotavirus is a double-stranded RNA virus, it belongs to Reoviridae family and the genotypes possess 11 genera (Fields, 1996). Eight genotypes of the virus have been distinguished and classified as A, B, C, D, E, F, G and H. Human beings are mainly affected with A, B and C, specie but A is the most common specie and causes beyond 90% of rotavirus infections in humans (Kapikian & Shope, 1996).

The Group A rotavirus has been genomically classified further through a sequence-based system which permits for the viral genome segment to be consigned a particular genotype. RVA's are an important cause of diarrhoea of new-borns in humans and several animal species, including calves (Theil, 1990). RVs are distinguished by the neutralizing antigens of their structural proteins (VP4 and VP7), and the classification

has engendered the G type and P type serogroups respectively. (Hoshino and Kapikian, 1994).

Previously, these types were based on serological classifications, however progressively, circulating G and P typing is now genomically oriented. Most of the serogroup G types and P types found circulating in most animal populations have been detected. This allows for the design of effective vaccines to control interspecies infections. The serogroups G and P specific types are usually associated with specific animal species. G1 to G4 are said to be the most commonly found in humans while bovine rotaviruses mostly belong to G types 6, 8, and 10 and these have P types 1, 5, or 11. Characterization from various locations worldwide has rendered host species specificity of the P and G types less distinctive. Genotypes G6, G8, and G10, which were initially classified as specific for cattle, are now being found in humans (Jagannath *et al.*, 2000). Considerable sequence heterogeneity has been detected in two of the viral non-structural proteins, NSP1 and NSP4, this allows for grouping those from the same animal species together. Bovine rotaviruses have their NSP1 clustered just as their NSP4s (Ciarlet *et al.*, 2000)

Phylogenetic analysis of the rotavirus genome has revealed recurrent associations between the development of human- animal strains which could possibly be as a result of numerous occasions of re-transmission among various animal species. Interspecies transmission and successive gene re-assortment are a significant mechanism controlling rotavirus diversity and allowing for the occurrence of new pathogenic strains (Moutelíková & Prodělalová, 2015).

Studies in Ghana have reported of new emerging strains of the virus in paediatric diarrheal cases some of which have been identified as animal genotype whilst others have been identified as re-assorted variants resulting from both human and animal rotavirus strains. (Enweruno-Laryea *et al.*, 2014). Investigations have proven that close co-living of humans and animals may have resulted in such interspecies infections due to recombination of the human and the animal variants of the rotaviruses, by close contact with animal faecal material (Amoah-Barnie, 2014).

The virus is spread by the faecal-oral route, through contact with contaminated hands, surfaces and objects. A possible respiratory route has been described by the South Australian National Health Services. They indicate that the virus can be spread by mucous membrane of many parts of the body such as the nose, mouth, throat and genitals thus contact with infected airborne droplets by coughing and sneezing could transmit the virus, (South Australian National Health Services Directory, 2018). The virus is highly transmissible; an infected person's faeces may contain more than 10 trillion infectious particles per gram, however less than 100 particles are enough to transmit the infection (Grimwood & Lambert, 2009; Bishop, 1996). The WHO suggests, the disease is a manageable childhood disease however over 450,000 children under five years of age die from the infection each year, mostly in developing countries, while two million more become severely ill (Tate *et al.*, 2012; WHO, 2008).

In Ghana, most peasant farmers and pet owners live in close proximity to their animals especially in the same compound having kraals in the middle of the compound house system, a common practice that provides the basis for human –animal cross infection. In some cases, the entire community shares the same water source with their animals, with

the latter having direct access to the water source just as the humans. In such situations, faecal materials as well as nasal discharges are spread in the household setting and the water source, which are ideal to promulgate the easy spread of the virus to and from animals to humans.

This situation is compounded by wrong diagnosis of diarrhoea cases in livestock where rotavirus infections are misdiagnosed for Peste des Petit Ruminant infection (PPR infections, also a viral infection) due to the singular presence of profuse diarrhoea in both cases and consequently obviating the other symptoms of the latter which in essence is occasionally presented in animals with scouring diarrhoea. This tends to aggravate the rapid spread of the disease amongst other livestock and children in the house hold alike. It is also reported that the disease which occurs all year round in Ghana, has its seasonal peak occurring during the cool dry months of the year (Armah *et al.*, 1994). Rotaviruses are said to be quite stable in nature and are found up to levels of 1-5 infectious particles in estuary samples and they can be stable for between 9-19 days. Hygienic measures used to eradicate bacteria and parasites are ineffective in the control of rotavirus. As such, the incidences of the infection in countries with high and low health standards are the same (Khatib &Khan, 2013).

Gastroenteritis caused by rotavirus can be mild to severe and is characterized by low grade fever, vomiting and diarrhoea. Children will develop fever, followed by vomiting, then subsequently watery diarrhoea thus causing rapid dehydration in the patient. Once there is an infection, incubation period takes about two days before symptoms appear (Sharpe & Fields, 1985). The period of illness is acute usually starts with vomiting and subsequently four to eight days profuse diarrhoea. Dehydration with dizziness in

rotavirus infection is common than bacterial infections, and is the most common cause of death, (Soriano-Brucher *et al.*, 1991). Dizziness while standing, decrease in urination and dry mouth and throat form part of symptoms of dehydration associated with Rotavirus. Viral re-assortment and retransmission into the human population could have a long-term effect on rotavirus disease appearance or form of manifestation, diagnosis, treatment and vaccine efficacy for prevention, due to genotypic shift or drift and recombination.

RVA diarrhoeal disease is highest in low income countries, especially in Africa and Asia. An estimated 453,000 deaths occurred as of 2008 globally in children under 5 years of age with over 50% of these occurrences in Africa (Tate *et al.*, 2012). An estimated annual death of 310,000 in sub-Saharan Africa is associated to RVA gastroenteric infections. (Sanchez-Padilla *et al.*, 2009). World Health Organization (WHO) reports that, five African countries recorded mortality rates of more than 300 per 100,000 children under five years old (Tate *et al.*, 2012). Almost 320 deaths are reported in South Africa alone annually due to the infection (Sanchez-Padilla *et al.*, 2009).

1.2 PROBLEM STATEMENT

Contrary to the previous assertion that animal rotaviruses did not affect humans under natural conditions, many recent works have proven that interspecies transmission is possible under natural conditions (Jere *et al.*, 2014), either by gene segment resortment or as whole virions and this is said to be taking place at a relatively high frequency (Kapikian *et al.*, 1990). According to Bányai *et al.* (2012), globally, RV infections caused by mixed G and P genotypes increased from 7.9% to 11.7% between 1996 to 2007. Nyaga *et al.*, (2015) reported that mixed G and P genotype infections in Africa

were detected during a WHO-coordinated RV surveillance studies, with an overall detection rate range of 12% to 14%. Nyaga *et al.* (2015) also report an increase in the detection of mixed G and P genotypes in Africa, thus more investigations at the genome level is required to determine the properties of these mixed infections at the genomic level in order to understand their origin.

One of the settings under which such mixed genotype infections are possible are communities in which humans and animals live in close association. Examples are geographical areas where domestic animal husbandry is commonly practiced. In Ghana, very limited investigations have been conducted in this area. For example, Amoah Barmie, 2014 though on a small scale isolated several animal-human rotavirus recombinants in a few communities, where animals and humans live in close association in Accra.

In this current study, the investigation will be carried out on a larger scale in Akuse and its environs, where domestic animal rearing is practiced widely among livestock farmers. This will enable us determine if animal–human co-existence in close proximity could favour recombinant transmissions among the different species (animal-human) in that setting and how this can propel and aggravate the dilemma of possible public health implications in our Ghanaian communal settings where most animal rearing is done by the extensive method or free-range practices.

1.3 SIGNIFICANCE OF STUDY

In Ghana there has been a considerable decline in hospitalization of children of age 59 months and younger for diarrhoea, a phenomenon which is attributed to the introduction

the Rotarix vaccine (Enweruno-Laryea *et al.*, 2014). The decline of the diarrhoea syndrome offers a valuable and practical measure of vaccine impact on public health disease control and informative for policy decision making in the choice of vaccine formulation selection for rotavirus control.

With the gradual increase in the detection of mixed G and P genotypes in Ghana, more research at the whole-genome level is required to determine the characteristics of these mixed infections and its association with close proximity living between humans and their livestock population in the same environment. This will enable an informed selection of genotypes which can be incorporated into future vaccine formulations for human and animal use as well as the stability of the current vaccine being circulated in the population. Results from this scientifically relevant research is important considering the current public health implication of the disease in the country in spite of the vaccination programs established. Also reports from other researchers indicate that RV whole genome sequences being reported from Africa are relatively low compared to those from other parts of the world (Jere *et al.*, 2014; Magagula *et al.*, 2015, Jere *et al.*, 2014; Magagula *et al.*, 2015). Such information will enable us understand viral factors that allow for the emergence of more pathogenic strains, and this could possibly enhance our understanding of the lower immunogenicity of the vaccine response and the efficacy of RV vaccines observed in developing countries such as Ghana (Armah *et al.*, 2010).

Surveillance for diarrhoea cases in livestock is not routinely practiced in the veterinary sector in Ghana, there is therefore very little data available to account for the seasonal scouring diarrhoea in calves, lambs and piglets (small ruminants). Even though RV is recognized as a major diarrhoea infection in livestock in Ghana, detailed laboratory -

based investigation of the disease is very limited. Therefore, very little is known about the different genotypes circulating among livestock in Ghana. Nakagomi *et al.* (2012) stated that, there is a constant flux of genetic materials among co-circulating human and animal rotaviruses and the segmented nature of the virus provides the opportunity for re-assortment between these co-circulating strains. For these reasons, identification of the genotypic variations of the current rotavirus viral recombination circulating in our communities will provide reliable data to measure the practical efficacy and impact of current vaccines being used for public health disease control, and to influence decision making policies. Data from the study will also inform the need for the introduction of rotavirus vaccine such as Rotavec corona in our livestock industry for disease control and prevention.

1.4 AIM

To characterize genotypic variations in rotavirus isolates from humans and animals living in close association in communities in and around Akuse and its environs.

1.5 SPECIFIC OBJECTIVES

1. To determine the prevalence of rotavirus infection among study participants and the behavioural patterns and demographic characteristics which favour animal-human transmission in these communities.
2. To determine molecular characterization of rotavirus genotypes circulating among humans and animals in these communities.

1. 6 HYPOTHESIS

Communities where humans and animals live in close proximity could generate recombinant re-assortment of rotavirus strains.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 ROTAVIRUSES

2.1.1 Brief History

Gastroenteritis over the decades has been a leading cause of infantile death with various causes such as bacteria, parasites or viruses (Sai *et al.* 2013). However, until 1973, no particular pathogen could be detected in about 80% of patients admitted to hospitals with severe dehydrating diarrhoea, though acute diarrhoea had been a major worldwide cause of death in young children for centuries. In 1973, Ruth Bishop and colleagues detected abundant viral particles of a 'new' virus in the cytoplasm of mature epithelial cells lining duodenal villi and in faeces from such children admitted to the Royal Children's Hospital, Melbourne (Bishop, 1996). Since that period, rotavirus gastroenteritis has been implicated as the main cause of acute gastroenteritis in humans and animals (Parashar *et al.*, 2006). Related viruses, found in several other species of animals causing acute gastroenteritis, were described in 1976 (Wood *et al.*, 1976). These were also recognised a group of pathogens affecting humans and animals worldwide (Wood *et al.*, 1976; Iewe *et al.*, 1974).

Initially, several names were given to the 70-nm virus and these include, reovirus-like, orbivirus-like, duovirus, infantile gastroenteritis virus, or a 'new' virus and the wheel-like structure visualised by electron microscopy. However, the Rotavirus was described as wheel in 1974 by Henry Thomas Flewett (Flewett & Woode, 1978), meanwhile the serotypes were said to be first described in 1980, and in the subsequent year, rotavirus from humans was first grown in cell cultures. Eventually this led to an agreement to accept the name Rotavirus (rota = Latin for wheel) which became the internationally

accepted taxonomic name derived from monkey kidneys, by adding trypsin (an enzyme found in the duodenum of mammals and now known to be essential for rotavirus to replicate) to the culture medium (Ward & Bernstein, 2009).

2.1.2 Structure

The wheel like appearance of Rotaviruses has a defined rim radiating with short spikes (Fig. 2.1) (Angel *et al.*, 2007). The entire viral particle is made up of an inner core, an intermediate capsid and an outer capsid from which the short spikes emanate (Estes & Cohen, 1989). Their ds- RNA genome consists of 11 genome segments which codes for one protein each except the 11th segment which codes for 2 different non-structural proteins (NSP5 and NSP6) in some strains (Estes & Cohen, 1989). Six of the gene segments encode viral structural proteins (VP1–VP4, VP6 and VP7) which are in the virion and the other six non-structural proteins (NSP1, NSP2, NSP3, NSP4 and NSP5/NSP6) which are produced only in infected cells (Estes, 2001).

The rotavirus structure consists of an inner VP2 protein layer which delimits the RNA segments and other molecules such as the VP1 and VP3 proteins (Lu X *et al.*, 2008). In the middle is the VP6 capsid protein and the outer layer contains the VP4 protein spikes which is embedded in a VP7 capsid. The VP4 and VP7 are composed of neutralizing antigens necessary for viral entry and infection of target cells. The outer capsid is mostly formed from the VP7 which surrounds the virion. In the presence of trypsin-like protease, the VP4 is cleaved into VP8 and VP5 (polypeptides) (Patton, 2012). The VP8 forms the head whilst the VP5 forms the stalk base of the VP4 protein.

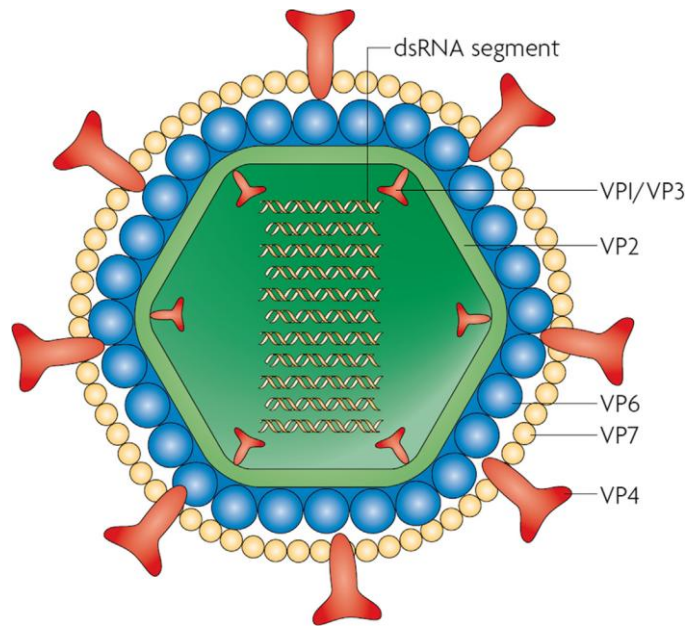


Figure 2.1: A schematic diagram of rotavirus virion (Angel *et al.*, 2007).

(The virion consists of an inner VP2 protein layer surrounding the RNA segments and several molecules of VP1 and VP3 proteins, a middle VP6 protein capsid, and an outer layer containing VP4 protein spikes embedded in a VP7 capsid (Angel *et al.*, 2007).

2.1.2.1 Structural proteins

2.1.2.1.1 Core proteins

The core proteins are made up of the VP1, VP2 and VP3 protein particles which form the structural proteins of the virus (Fig. 2.1). The VP1, an RNA dependent viral RNA polymerase with a size of 125 kDa, has a sequence specific recognition which aids in viral RNA binding. All the core proteins participate in the process of viral RNA transcription and replication, the VP1 interacts with VP2 and VP3 and functions both as a transcriptase and replicase (Varani & Allain, 2002).

The innermost structural part of the viral capsid is the VP2 with a size of 95 kDa. VP2 acts as replication intermediate while VP3 acts as a capping enzyme and functions as a

guanylyl and methyl transferase coupling in the binding of single stranded RNA. It has a mass of 88 kDa (Vende *et al.*, 2002).

2.1.2.1.2 Inner capsid protein

The inner capsid is mainly formed by the VP6 (Fig. 2.2), the middle/intermediate layer and is by the gene segment 6. VP6 has a molecular weight of 45 kDa (Estes *et al.*, 1987). It interacts with the outer capsid proteins VP4, VP7 and the core protein VP2. Even though extremely stable, these form spontaneous trimmers which can be disassociated and reassembled by pH changes (Tosser *et al.*, 19992). The VP6 is hydrophobic, highly antigenic and immunogenic, thus a major antigenic detection target for rotavirus diagnosis in stools. It is also used to classify the rotavirus into subgroups (Gorziglia *et al.*, 1988).

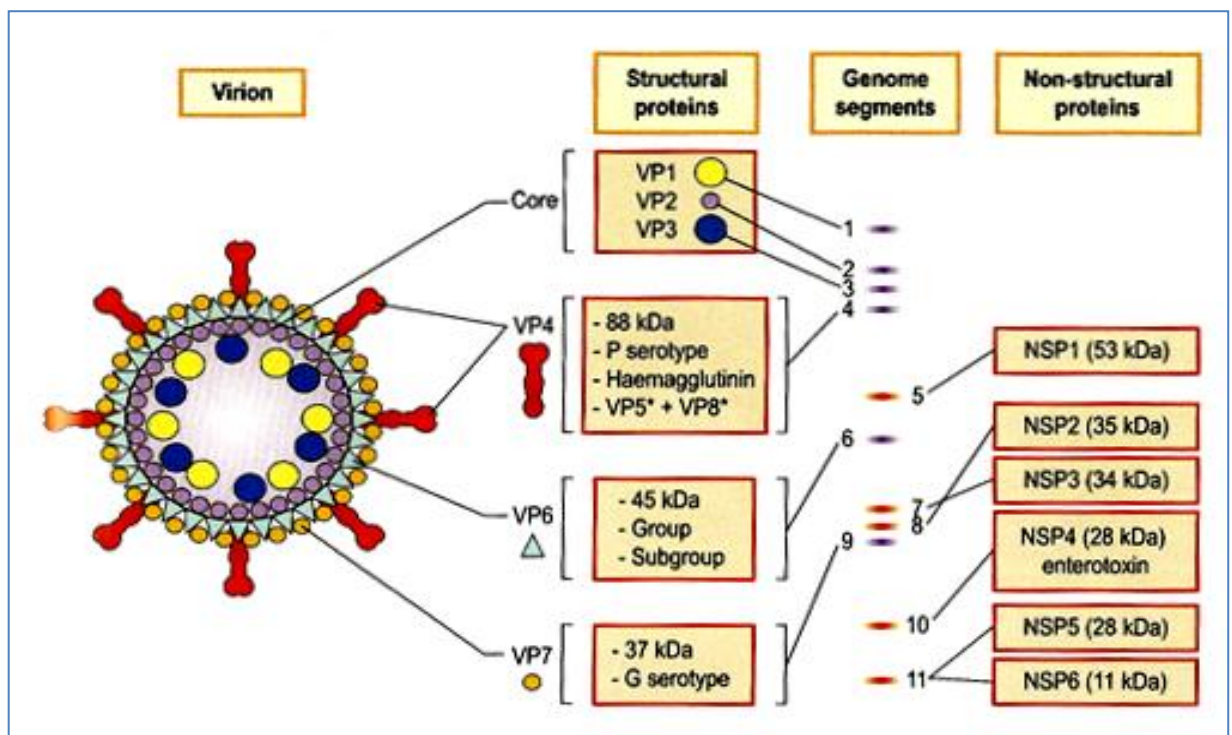


Figure 2.2: A simplified diagram of the location of rotavirus structural protein (Patton, 1995).

2.1.2.1.3 Outer capsid protein

The outer capsid of a mature rotavirus particle is composed of VP4 and VP7 and weighs about 34 kDa. The VP4 is a non- glycosylated protease sensitive series of 60 short spikes, approximately 10 to 12 nm in length that projects from the outer capsid (Estes & Greenberg, 2016). VP4 is an integral membrane glycoprotein and a neutralizing antigen thus it is used in the classification of rotaviruses into G genotypes and serotypes (Ciarlet & Estes, 2001; Pesavento *et al.*, 2006).

The VP4 has a knob like structure at its distal end containing proteolytic cleavage product VP5* (Zang *et al.*, 2000). VP4 has a molecular weight of 88 kDa and constitute 1.5% of virion protein (Mattion *et al.*, 1994).

Viral infectivity and penetration into target cells is enhanced by VP4 cleavage into VP5* and VP8*, with molecular weights of 60 and 28 kDa, respectively (Estes & Grenbreg, 2016). According to Mackow *et al.* (1990), VP4 induces neutralizing antibodies which confers immunity to animals as well as immunogenicity to children and animals alike (Conner *et al.*, 1998).

2.1.2.2 Non-structural proteins

The non- structural proteins or NSPs are viral protein particles encoded by the viral genome in charge of viral replication and assembling for the release of matured virions (Hu *et al.*, 2012). They comprise of NSP1-NSP6 and their functions are specific even though the functions the NSP1 and NSP6 are not exactly clear. The functions of the NSPs have been defined as follows:

- i. NSP1 is involved in the repression of interferon regulatory factor 3 which inhibits host cell counter reaction to rotavirus infection (Graff *et al.*, 2002). Hu *et al.* (2012), reports the involvement of the NSP1 in viral growth by repressing the host cell apoptosis, thus rendering ineffective the innate cell immune responses.
- ii. NSP2 with the aid of the NSP5 plays a pivotal role in viral synthesis and packaging, thus the formation of endoplasmic reticulum or viroplasm in the host cell. Graff *et al.* (2002), states that the NSP2 is a replication intermediary. It is said to exhibit kinase activities and important in the binding of single stranded RNA.
- iii. NSP3 promotes viral protein synthesis by binding to the viral mRNA at the 3' end and also represses host cell protein synthesis (Graff *et al.*, 2002).
- iv. NSP4 has been reported as the enterotoxin responsible for the occurrence of diarrhoea during rotavirus infection (Mandell *et al.*, 2007; Srivastava & Jain, 2015; Yaqoob *et al.*, 2016). Iturriza-Gómara *et al.* (2003), reports of a correlation between the NSP4 genotype and the VP6 virus subgroup. The NSP4 is said to induce the diarrhoea through binding to specific receptors which essentially triggers the phospholipase C signalling pathway. This induces the release of an increased amount of calcium ions and activates an increased release of chloride ions resulting in conformational change in the intestinal microvilli thus causing malabsorption and subsequently diarrhoea. Other systems such as the enteric nervous systems (ENS) are also triggered (Greenberg & Estes, 2009).
- v. NSP5 binds to NSP2 in the process of viroplasm formation.
- vi. The precise role for NSP6 is, however, not known except for its involvement in viral replication.

2.1.2.3 Structural Proteins of Rotavirus

Under the electron microscope, rotaviruses can be identified morphologically as:

- i. Single shelled particle: This is a viral particle devoid of the outer capsid structure. It forms a circular brittle appearance measuring between 50-65 nm in diameter. It contains the major structural proteins (Tosser *et al.*, 1992).
- ii. Core particle: Devoid of an outer and inner capsid; it measures between 37- 48 nm in diameter (Prasad *et al.*, 1990). Prasad *et al.* (1988) reported that the core particle is composed of VP2 protein with a hexagonal structure under the electron microscope.
- iii. Complete infectious particle: This is a non- enveloped complete infectious particle which contains the core, inner and outer capsid. It consists of a double shelled icosahedral protein capsid and measures 70 nm in diameter (Prasad *et al.*, 1990).

2.1.3 Classification

Rotaviruses are categorized as a distinct genus from the members of the Reoviridae family due to the similarities in the structure of their capsid, genomic conformation, organization, and replication strategy (Varani & Allain, 2002; Gosg *et.al.*, 2012). Amongst the Reoviridae family are the Coltivirus, Oryzovirus, Aquareovirus, Orbivirus, Phytoreovirus, Othoreovirus, Cypovirus and Fujivirus (Kapikian & Channock, 1996).

Further differences based on their antigenic properties, gene sequences and genome pattern within the genus have been used to classify the genus into serogroups, subgroups, serotypes, genotypes and electropherotypes. The group and subgroup specification is solely based on the inner shell protein VP6. The terms serotypes and

genotypes are solely used dependent on if the method of detection is based on the antigen (MAb-ELISA and cross-neutralization assay) or nucleic acid-based detection methods (nucleotide sequencing, RT-PCR, and oligonucleotide probe hybridization) (Estes *et al.*, 2013). However, the P serotypes and P genotypes are designated differently because they are more in numbers than the serotypes and genotypes belonging to the G and are used interchangeably as there is a concordance between genotype and serotype (Gray *et al.*, 2008). However, methods for rotavirus classification has evolved from antibody based to genetic characterization, offering a nucleotide sequence identification for each of the RVA (11 segments) segments (Esona *et al.*, 2010).

2.1.3.1 Serological and Genotypic

Rotavirus classification is based on the properties of the two outer capsid proteins VP7 (glycoprotein, G-types) and VP4 (protease sensitive, P-types) serotypes and genotypes (Matthijnsens *et al.*, 2011). Trojnar *et al.* (2013) have described 27 G and 37 P types. Of these only 12 G and 15 P genotypes are said to infect humans (Esona *et al.*, 2010; Matijnsen *et al.*, 2011).

G serotypes are further segregated into 4 subgroups (SGI, SGII, I+II, and SG non I/non II) all based on reactivity patterns with monoclonal antibodies specific to the subgroup (MAbs) directed at the VP6 (Svensson *et al.*, 1998; Esona & Gautam, 2009). Human rotaviruses are known to belong either subgroup I or subgroup II or both. These are distinguished on the basis of VP6 diversity (Greenberg *et al.*, 1983).

Rotaviruses may also be classified according to their genome patterns in the polyacrylamide gel as long electropherotype or short profile electropherotype (E-type)

(Matthijnsens *et.al.*, 2012). Their differences are found in the migratory patterns of the genome segments 10 and 11 on the polyacrylamide gel. There are eight groups designated according to their migration pattern on polyacrylamide gel (from A–H) (Table 2.1). A–H groups have been identified (Matthijnsens *et al.*, 2012). Of these, four (A, B, C and H) are responsible for the rotavirus infection in humans. The A viruses cause 90% of the infection in humans which also affects animals (Nyaga *et. al.*, 2016).

Currently, not less than 27 G, 37 P, 16 I, 9 R, 9 C, 8 M, 16 A, 9 N, 12 T, 15 E, and 11 H genotypes have been identified from humans and/or animals (Esona Mathew&Rashi Gautam 2015). In recent times P and G type combination has been found in humans and animals alike. Amongst them are G1P [8], G2P [4], G3P [8], G4P [8] and G9P [8] (Esona *et al.*, 2015). The emergence of strange and unusual strains has led to the formation of atypical and untypable rotavirus strains especially in mixed infections (Esona *et. al.*, 2015).

Genetic re-assortment due to direct animal to human transmission has culminated in a larger classification system that involves all 11 segments of the virus genome (Park *et. al.*, 2011). The Rotavirus Working Group has assigned the current classifications to the genotype of each of the 11 rotavirus proteins as follows: G_x-P_[x]-I_x-R_x-C_x-M_x-A_x-N_x-T_x-E_x-H_x for VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 (Matijnssen *et al.*, 2011).

Table 2.1: Host range of antigenic groups (Barnie *et.al*, 2010).

Rotavirus Group	Host Reservoir
A	Human, primate, pig, cat, rabbit, cow, bird, mouse
B	Human, sheep, cow, rat
C	Ferret, human, pig
D	Chicken
E	Pig
F	Chicken
G	Chicken
H	Human

2.1.4 Viral Replication and Pathogenesis

2.1.4.1 Viral replication

Replication of the virus occurs in the cell cytoplasm where the viral mRNA is formed using viral RNA polymerase to form structural units of the capsid (Estes &Greenberg, 2016).Segments are assembled into immature capsids to form double stranded RNA genomes.Within 24 hrs of infection, a great number of viral particles are released in the stool and the intestinal villus (epithelium) changes from columnar to cuboidal shape, becoming stunted and shortened (Lundgren & Svennson, 2001).Severe morphological changes are mostly observed in the upper portions of the small intestine and there could be little or no inflammation. The severity of these changes relates to the severity of the illness subsequently (Lundgren & Svennson, 2001).

The virions, having escaped the acidic environment in the stomach due to their triple capsid protein, proceed to invade the enterocytes of the intestinal villi by a process

known as endocytosis. This causes functional and structural changes of the epithelium with the formation of an endosome (viroplasm) (Greenberg *et al.*, 2009) mRNA for both protein biosynthesis and gene replication are produced during the infection process, this aids in the packaging and replication of the ds-RNA in the cytoplasmic inclusion bodies (Patton *et al.*, 2006).

2.1.4.2 Pathogenesis

Rotavirus particles infect intestinal enterocytes through faecal–oral transmission and this is mediated by virus-epithelial cell interactions in the middle and upper portions of the small intestinal villi, thus causing diarrhoea (Estes & Greenberg, 2016). However, there are other host–virus factors which influence the outcome of the disease. The most plausible host–virus factor which affects the disease outcome is the age (Esona & Gautam, 2015). Children above three months of age become vulnerable due to the waning of the titers of the maternal antibodies and the age of exposure to the infection (Esona & Gautam, 2015; Greenberg *et al.*, 2009). In spite of this several other mechanisms e.g. viral attachment, cell entry, formation of endosomes or viroplasms, viral replication, transcription, translation and cell lysis or release, aid in the induction of the disease.

2.1.4.2.1 Attachment

The viral attachment process consists of uncovering of the viral protein in the digestive tract by protease digestion which removes some or all the outer capsid protein (VP4). Ruiz *et al.* (2009) reports that attachment is mediated by the VP4 and VP7. The virus invades target cells either by direct entry or fusion with enterocytes and through Ca⁺ dependent endocytosis (Lundgren & Svensson, 2001). The binding and entry of the virus

into the host cells is thought to be a multifactorial process (Lopez & Arias, 2004). This mechanism causes malabsorption due to the destruction of enterocytes and their absorptive functions, villus ischemia, flow of intracellular fluid and chloride ion by NSP4 and stimulation of enteric and vascular systems causing indirect secretions (Esona & Gautam, 2009).

2.1.4.2.2 Cell entry and binding

Infection of the enterocytes by the virus begins with the entry of the virus, subsequent uncoating, transcription, translation of viral proteins, formation of endosomes (Vi) and release of virus. Virions are released with intracellular NSP4 (red arrows) which induces Ca^{2+} release which flows from the endoplasmic reticulum (Fig 2.3a). The NSP4 allows water and electrolytes to enter the intestinal lumen (arrows). It binds with specific receptors and triggers several signalling cascades through phospholipase C (plc) and inositol (ip3) which increases calcium in the system and consequently the destruction of microvillar cytoskeleton (Fig 2.3a & c) (Estes & Greenberg, 2016).

The NSP4 can act directly on an intestinal crypt (brown) or induces the secretion of Cl^- (chloride ion) which stimulates the enteric nervous system (ENS) thus signalling Ca increase. Some animal rotavirus strains require sialic acid (SA, N-acetylneuraminic acid) residues on the surface of target cells for initial attachment (Superti and Donelli, 1991). Such rotaviruses are sensitive to neuraminidase which greatly diminishes their infectivity by the treatment of neuraminidase.

Numerous strains from animals and most isolated strains from humans are neuraminidase-resistant (Ciarlet & Estes, 1999). Sialic acids in oligasaccharide structures

are less sensitive or non-responsive to neuraminidase however it does not mean that these strains do not use sialic acid for cell attachment. (Delorme *et al.* 2001). There is a probable conformational change in the VP4/VP7 due to their interaction with sialic acid which allows the virus to then interact with the integrin $\alpha 2\beta 1$. Binding of the virus to the host cell eventually leads to infiltration and uncovering of the virus (removal of VP4 and VP7 proteins from the virus) to yield the transcriptionally active double-layered particles).

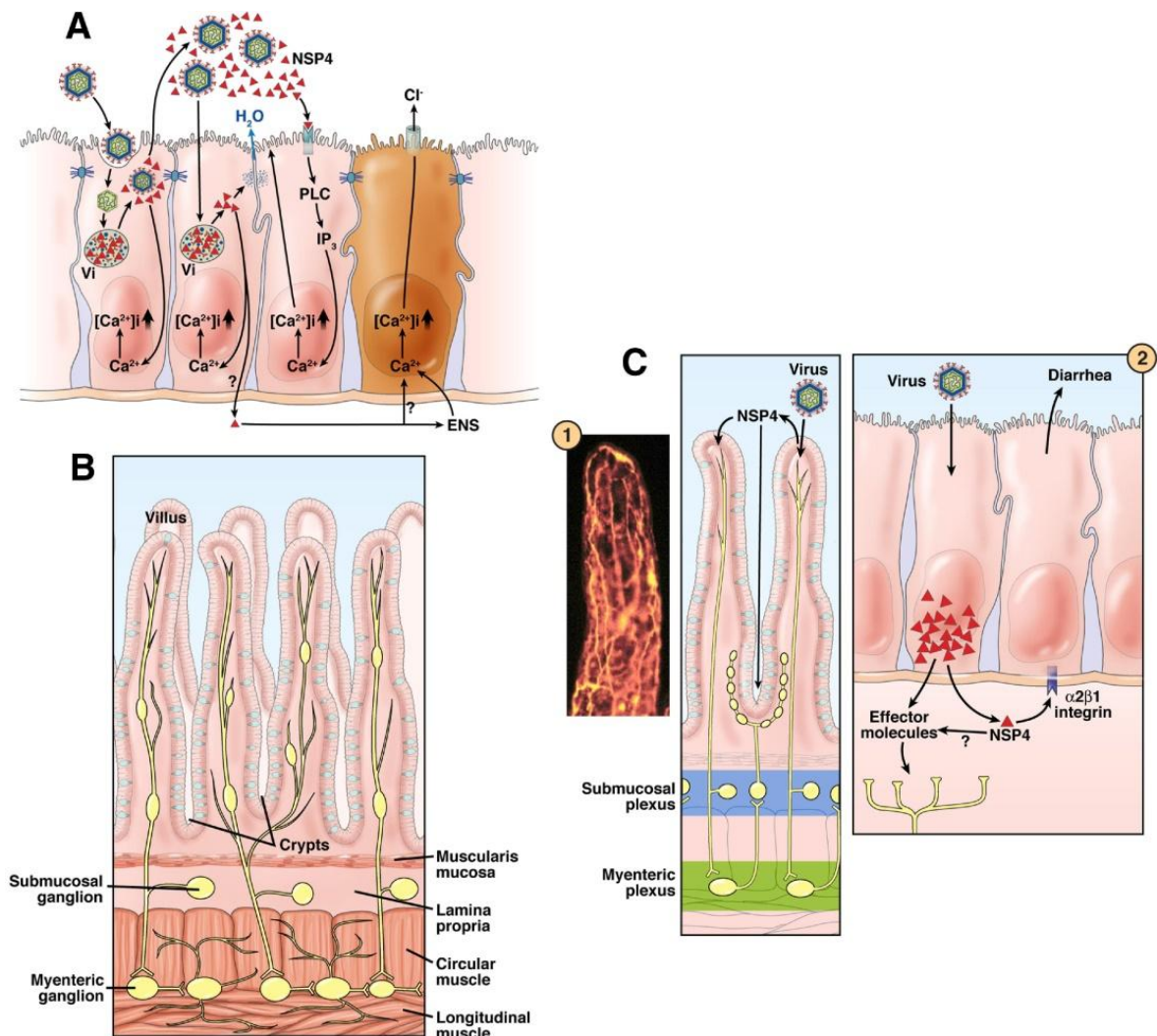


Figure 2.3: Mechanisms by which rotaviruses cause diarrhoea (Estes & Greenberg, 2016).

2.1.5 Epidemiology

The UNICEF and World Health Organization, 2009 report estimates that rotavirus causes 40% of all hospital admissions in children under five years of age worldwide due to diarrhea leading to some 100 million episodes of acute diarrhoea which results in 350,000 to 600,000 child deaths each year.

More than 90% of gastroenteritis in humans is caused by the rotavirus A (Leung *et al.*, 2005). It is endemic worldwide, there are almost 2 million reported cases each year in developing countries of diarrhoea, resulting in hospitalization (Rheingans *et.al.* 2006). Hospitalization results in an estimated 453,000 deaths of a child younger than five (Tate *et al.*, 2012), 85% of deaths are accounted for in the developing countries. According to World Health Organization (2008), prior to the vaccination programme in the United States alone, over 2.7 million rotavirus gastroenteritis occurred annually, with 60,000 children hospitalised and about 37 deaths as a result of the infection (Fischer *et al.*, 2007). The infection mostly occurs in the cool dry seasons. The number attributable to food contamination is unknown (Atchison *et al.*, 2010).

Fang *et al.*, (1989) reported of major epidemics of severe diarrhoea caused by the Rotavirus B, also called adult diarrhoea rotavirus which affected thousands of people of all ages in China as a result of contamination of drinking water by sewage. Rotavirus B infections also occurred in India in 1998, the causative strain was named CAL. Unlike the known rotavirus B, the CAL strain is said to be endemic in the area (Kelkar & Zade, 2004). Rotavirus B epidemics are said to be confined in main land however surveys indicate there's a lack of immunity to these species in the United States (Penaranda *et al.*, 1989).

Rotavirus C has also been associated with rare and sporadic cases of diarrhoea in children, where small outbreaks have occurred in families (Iturriza-Gómara *et al.*, 2004).

2.2 ROTAVIRUS DIARRHOEA

Rotavirus infection causes mild to severe gastroenteritis characterized by fever (low grade), vomiting and diarrhoea. Children will develop the triad of fever, followed by vomiting, then subsequently watery diarrhoea thus causing rapid dehydration in the patient (Patton, 2012). Resulting diarrhoea from rotavirus infection leads to weight loss, dehydration and death (Patton, 2012). Patton (2012) estimated approximately 1500 deaths daily and most affected by the disease are children under the age of five years as also indicated by Wardlaw *et al.* (2010).

The young of animals may also be infected with rotavirus leading to rotavirus diarrhoea which causes great economic losses to the livestock industry (Kobayashi *et al.*, 2007). Once an infant is infected, (human/animal) the incubation period last for about two days before symptoms appear (Sharpe & Fields, 1985; Chien Chen *et.al*, 2012).

2.2.1 Incubation Period

The period for incubation especially in young children is 24 to 78 hours after infection whereas it expands from 1 to 4 days. However, the infection can last averagely from 3 to 7 days whilst more severe cases can last up to 14 days (Bishop *et al.*, 1996; Esona *et al.*, 2015).

Rotavirus infection extends from subclinical infection to mild watery diarrhoea of a limited duration advancing to severe diarrhoea, vomiting and fever that can result in dehydration with shock, electrolyte imbalance, and death (Bishop *et al.*, 1996).

2.2.2 Symptoms

The symptoms often begin with vomiting in the acute period of illness, followed by 4 to 8 days of profuse diarrhoea with 10-20 bowel movements per day, electrolyte imbalance and metabolic acidosis (Estes & Greenberg, 2016). The infection may last longer in children who are immunocompromised due to congenital immunodeficiencies (Esona *et al.*, 2015). Most common in rotavirus infection is dehydration rotavirus infection than in most diarrhea caused by bacterial pathogens and is the most common cause of death related to rotavirus infection. (Soriano-Brucher, *et al.*,1991; Zuccotti *et.al*, 2010). Dehydration is most severe in young infants and young children ((Iturriza-Gómara *et al.*, 2000, 2004).

Other signs of infection include dizziness while standing, reduction in urination, dry mouth and throat, irritability, lethargy, pharyngeal erythema, rhinitis, and palpable cervical lymph nodes, all superimposed by the diarrhoea (Esona *et al.*, 2015). Rotavirus proteins and RNA have been detected in several organs, including body fluids such as blood and cerebrospinal fluid, though the clinical significance of these findings is not clear (Esona *et al.*, 2015; Blut *et al.*, 2007).

2.2.3 Treatment

The main approach of treatment in rotavirus infection is fluid therapy and replacement of electrolytes lost through vomiting and diarrhoea. However, this is sometimes

insufficient and as such the introduction of rotavirus vaccine has brought a great advancement in the campaign to reduce deaths caused by rotavirus (Path, 2012). Mild infections can be effectively treated like any other diarrhoea infection by administering fluids, salts and zinc tablets until the disease runs its course. Severe cases however require intravenous fluids lest they die of dehydration and or acidosis (Zuccotti *et.al*, 2010); however, the introduction of the vaccine has improved the campaign to reduce the disease burden.

As estimated by the WHO in March 2006 the number of deaths caused by rotavirus annually was 527,000; recently in 2013, the WHO now estimates that the death toll by rotavirus is 215,000, there has thus been a great decline in the child deaths due to the vaccine programs established. Nevertheless, there is still the need to strive towards the eradication of this disease.

2.2.4 Preventive Measures and Control

Globally, rotavirus infection is considered a problem if not managed properly, considering the emergence of more pathogenic strains of the virus which has a high attack rate like the (P6) G9 strains circulating in the Netherlands (Woddowson *et al.*, 2002). Due to the enormous ability of the virus to sustain its viability for longer period of time outside the body, in the environment and surfaces and readily cause infection, vaccination provides the only plausible means of prevention and control. Adequate sanitary measures for eliminating bacteria and parasites seem to be ineffective in the control of the rotavirus. Thus, the incidence of rotavirus infection in countries with high and low health standards are the same (both in developing and developed

countries), as such their viability does not change with improved sanitary condition (Ahmed *et al.*, 2009).

Viral particles are easily transmitted in day cares centres and hospital settings where the virus is released at very high concentrations in stools of infected patients. Recommended control measures include isolation of the sick, cleaning of surfaces and hands with agents containing alcohol, Virkon, water quality control and improvement appropriate disinfection of environmental surfaces with prescribed detergents to maintain good hygiene and sanitation (UNICEF and World Health Organization, 2009).

Global interventions for control and prevention of the disease includes the introduction of vaccines such as Rotashield which was licensed in 1998 but was withdrawn in October 1999 due to complications such as an elevated risk of intussusception in the children. (Vesikari,2008).

Rotatrix and Rotateq are the major vaccinary control for the virus, however they are known to provide less than 100% protection against circulating strains in humans with the animal viral strains widely over looked in most of the third world countries including Ghana. Till date vaccination provides the only plausible means to reduce the associated morbidity, mortality and economic loss caused by the Rotavirus infection.

Current vaccines such as Rotateq by Merck and Co and Rotatrix developed by Glaxo Smith Kline 2008 respectively are said to have shown an efficacy of 72.5% to 100% against all rotavirus infections in humans. Rotateq with a 73% efficacy whiles Rotatrix

offers a 90% coverage against general rotavirus episodes and severe gastroenteritis in humans (Vesikari, 2008).

Vaccine interventions and treatment methods including exclusive breast feeding in both developed and developing countries has offered the most cost-effective reduction in morbidity and mortality for public health control and prevention of the disease. The main objective of the vaccine is to prevent the infection at least during the first three years of the child's life when the viral impact is highest (Velasquez *et al.*, 1996).

In Ghana, rotavirus is said to cause approximately 2090 deaths in children under the age of five annually (Path, 2012). However, with the assistance of the Global Alliance for Vaccines (GAVI) organization, the Rotarix vaccine was introduced in 2012 and incorporated into the National Immunization program (Dennis *et al.*, 2014). It is administered in two doses within a one-month interval where the initial dose is given at six weeks after birth. (Glaxo SmithKline Inc., 2014).

There is the challenge of unusual emerging strains due to genetic re-assortment and zoonosis that poses a huge health risk. It is thus a global concern and must be studied to reduce the disease burden as well as the interspecies transmission. Viral re-assortment and retransmission into the human population could have a long-term effect on rotavirus disease appearance or form of manifestation, diagnosis, treatment and vaccine efficacy for disease control and prevention, due to genotypic shift or drift and recombinations.

2.2.5 Conferred Immunity:

It is reported, that recovery from a rota virus infection does not always confer immunity to the patient, especially in a first-time infection. However, children under 3 months of age usually do not develop symptoms of diarrhoea due to immunoglobulin G which is a rotavirus specific trans- placental maternal antibody. (Ray *et al.*, 2007; Esona & Gautam, 2015).

Symptomatic infections typically occur at 3 months and older when maternal antibodies have waned off, thus making primary infection most severe. However, repeated recurrence of the infection confers protective antibodies against severe infection and recurrence of infection affects all ages and confers more protection in severe infections (Esona & Gautam, 2015)

2.3 ROTAVIRUS DETECTION AND DIAGNOSIS

The success in the treatment of a rotavirus infection depends on the ability to detect the virus early enough to allow for the appropriate treatment. Different serologic and molecular methods have been employed in the quest for rapid detection, management and control of the disease and, amongst these are:

- i. Enzyme immunoassays (EIAs) ELISA, Latex Agglutination has a sensitivity range of 70% to 98% and specificity range of 71% to 100% (Bernstein, 2009).
- ii. The PAGE or polyacrylamide gel electrophoresis of viral RNA segments,
- iii. Virus isolation in cell culture (Bailey *et al.*, 2013).
- iv. Electron microscope (EM). (Moutelikova *et.al.*, 2016)
- v. Immunochromatographic tests.

Currently, there are multipathogen detection assays rapid and sensitive in the detection of various enteropathogens like rotaviruses, other viruses, bacteria and parasites. Recent serologic and molecular methods for viral genotyping, strain characterization and serotyping have proven more sensitive, rapid and reliable for the detection of rotavirus strains. Some of these include:

- i. Dot blot hybridization, (Yamakawa *et al.*, 1989; Esona & Gautam, 2009), RT-PCR followed by gel-based genotyping, real-time RT-PCR, qRT-PCR, (Kottardi *et al.*, 2012),
- ii. Sanger sequencing, for full-genome sequencing (Fischer *et al.*, 2004).

2.3.1 Rotavirus Detection Assays

2.3.1.1 Electron microscopy

The electron microscope offers a more direct morphological diagnosis using stool samples to detect and confirm the presence of the rotavirus (Monini *et al.*, 2014; Moutelikova *et.al.*, 2016). It is specific, faeces are stained with phosphotungstic acid to detect the viral particles by its characteristic appearance of triple layer virus. However, it does not distinguish between a group A and non- group A rotavirus, it is very expensive and requires especially highly skilled personnel, thus it is not convenient for routine diagnosis (Saif *et al.*, 1980).

2.3.1.2 Cell culture isolation

Rotaviruses are cultured and isolated in African green monkey kidney cells and continuous cell lines. Infected cell is lysated using cesium chloride gradients, once purified virion is analysed sodium dodecyl-PAGE for verification of viral recovery or plaque assay can be used to determine viral titer in plaque forming units/ml based on

the cytopathic effect of the virus on the cell culture. Other alternative is the fluorescence focus assay which detects the viral antigen enabling viral quantification by the use of polyclonal antibodies engendered against the intact virions that cross-react with the viral strains thus producing a strong fluorescence signal. This technique is limited by the numerous rounds of passages in order to adapt and grow the virus *in vitro* thus making the process prone to contaminants. It is not a routine diagnostic method as viral recovery efficiency from faeces is very low, and it is also time consuming and labour intensive.

2.3.1.3 Polyacrylamide gel electrophoresis

This method allows for the direct visualisation of nucleic acid segments of extracted viral particles by PAGE followed by subsequent silver staining. Electrophoretic patterns from PAGE analysis express the different segment arrangement pertaining to the different groups of rotaviruses. There are short and long electropherotypes in both human and animals. The short one has 11 larger segments and migrates slowly encoding the NSP5. This method is comparable to EM and ELISA in sensitivity, however it is very labour intensive and takes long hours (Samuel Raymond, 1964).

2.3.1.4 Enzyme immunoassays

Enzyme immunoassays are widely used methods in rotavirus diagnosis, they offer simple rapid and highly sensitive diagnostic tool in routine diagnosis for rotavirus detection in stool specimens. It uses broad reactivity antibodies against VP6 epitopes (Dennehy *et al.*, 1998). They are specific and sensitive, 10 to 100 times more effective than the other assays and optical density is measured using a microtiter plate reader.

Currently there are several commercially prepared kits available for rotavirus diagnosis from stool samples. Some recommended ones are: Premier Rotaclone (Meridian Bioscience, Cincinnati Ohio), ProSpecT (Oxoid, Basingstoke, Hampshire, United Kingdom), and Ridascreen (R-Biopharm AG, Darmstadt, Germany).

2.3.1.5 Passive particle agglutination tests

This consists of coated latex particles with rotavirus antibodies which react to form visible macroscopic aggregates in the presence of a rotavirus antigen. This method is direct, easy to perform and more rapid than EM and EIAs. It can be used in clinic emergency rooms by physicians.

These are commercially available for detecting rotavirus in stool samples, such as : Rotalex latex agglutination kit (Orion Diagnostica, Helsinki, Finland), the Slidex Rota-Kit (bioMérieux, Marcy-l'Étoile, France), the Pastorex Rotavirus (Biorad, Hercules, CA), the RotaScreen latex test (Mercia Diagnostics, Surrey, United Kingdom), Rotastat (Novamed Ltd, Jerusalem, Israel), Meritec-Rotavirus (Meridian Diagnostics, Cincinnati, Ohio), Virogen (Wampole Laboratories, Cranbury, New Jersey), and the Wellcome Latex test (Wellcome Diagnostics, Research Triangle Park, North Carolina).

2.3.1.6 Immunochromatographic tests

They are also called lateral flow tests or strip tests, and they are qualitative tests for rotavirus detection in faecal samples. Their principle is based on sandwich immunochromatography by which antibodies against RVA-specific VP6 protein are used as a diagnostic tool to detect rotavirus antigen in stool samples. They offer strips embedded with anti-human antibodies in the membrane strip which react with the

rotavirus antigen in the stool. Once captured by the recombinant antibody they produce a colour change line in the window of the test kit to indicate positivity of results. They are highly sensitive, more rapid and specific than the EIAs and latex agglutination assays and can be used as rapid test kits in the clinics and laboratories.

Commercially available kits include: IP-Rota V (Immuno Probe, Frederick, MD), Dipstick ROTA (Eiken Chemical, Eiken, Japan), Rota-Adeno (Sekisui Medical, Tokyo, Japan), SAS Rota Test (SA Scientific, San Diego, CA), and ASAN Easy Test Rota strip.

2.3.1.7 Reverse transcription–polymerase chain reaction

This method is a combination of reverse transcription and polymerase chain reaction, capable of detecting very low concentrations of rotavirus in stool samples. It is useful for the verification of intact rotavirus RNA in RNA extracts (Wild *et al.*, 1991). It can be performed as a singleplex or a multiplex in combination with gel electrophoresis or capturing real time fluorescence.

During routine diagnosis, the VP6 which encodes a group-reactive protein, and is highly conserved in all RVAs is targeted by a designed RT-PCR assay which is VP6 gene specific. This is subsequently followed by gel electrophoresis, allowing for the detection of a wide range of rotavirus strains in stool samples.

2.3.1.8 Quantitative reverse transcription–polymerase chain reaction (qRT-PCR)

The qRT-PCR is a highly sensitive method with a higher output, faster turnaround time and quantification of viral load making it more effective than the traditional RT-PCR. Several types of the sensitive real-time RT-PCR methods have been developed with the

aim of targeting the VP2, VP4, VP6, VP7, NSP3 and NSP4 genes to enable the detection of RVA.

2.3.1.9 Non-structural protein 3 (NSP3) assay for rotavirus detection

Compared to other regions of the RVA genome, the 3'end of the NSP3 is considered a highly conserved region of the RVA genome thus highly targeted for the detection of a wide range of RVA genotypes. The One-step qRT-PCR assays targets this region also using thermo stable *Thermus thermophilus* polymerase enzyme (rTth) (Aldhoch *et al.*, 2011). The NSP3 qRT-PCR is said to be 100% sensitive and 86% specific. With the NSP3 qRT-PCR using a ds-RNA transcript allows for the quantification of the viral load in the stool sample, serum or cerebrospinal fluid. It is highly specific, sensitive and a broad-spectrum tool for the detection of various RVA genotypes. (Pang X *et al.*, 2011; Esoana & Gautam, 2009).

2.4 PORCINE ROTAVIRUS

Evidently, the rotaviruses are very ubiquitous with a wide range of host susceptibility thus various studies over decades have isolated and characterized the enteric pathogen which infects both man and animals. Porcine rotaviruses have been isolated, identified and characterized mostly in pigs (Cook, 2004).

Group A rotaviruses are the most important cause of rotavirus diarrhoea, it has been found causing rotavirus diarrhoea and non- diarrhoea infections in pigs associated with other enteric pathogenic agents. They are usually found in weaned piglets or recently weaned between the ages of 1-8 weeks resulting in devastating economic losses in the pig industry (Martella *et al.*, 2010). Will *et al.* (1994), detected that the infection is

mostly found amongst nursing and weaning pigs causing a rate of mortality and morbidity in this age group of animals. They further state that rotavirus can be detected in healthy piglets and virulence could be affected by factors such as the infecting strain, the dose, environmental conditions, colostrum antibodies and other host factors that differ specifically from pig to pig. In their study, Will *et al.* (1994) also identified group A, B as well as C rotavirus in the pig faecal samples studied and concluded that the peak of infection was in February and later in August – September. Numerous studies in countries such as Nigeria have proven the importance and severity of the rotavirus infection on the piggery industry.

Zoonotic transmission could not be over emphasised in this situation due to the natural capacity of the pigs to harbour the virus and to transmit to humans in their close proximity. Kim *et al.* in 2012 suggest that pigs are being considered as reservoirs for emerging rotavirus infections, agreeing with Cook *et al.* (2004) and Martella *et al.* (2010) who emphasized the potential zoonotic nature of rotavirus. Kim *et al.* (2012) describe rotavirus as the major cause of neonatal porcine diarrhoea causing significant losses in the piggery industry.

Monini *et al.* (2014), report that the most common of the P types in porcine rotavirus infection are P [6], P [7], P [13], P [19] and P [23] and the G types include G 3-5, G9 and G11. They state that apart from the common genotypes, other strains have been sporadically identified which include G6, G8, G10 and G12 and for the P types, P [5], P [8], P [32] and P [34]. According to a study by Papp *et al.* in 2013, the most common P and G type combinations in pigs is G5P[7]. Aside group A, B and C rotavirus that have

been detected in pigs, a new group, group H has also been identified (Molinari *et al.*, 2015).

The rotavirus diarrhoea in pigs is similar to diarrhoea is characterized by a watery diarrhoea which expands from a few days to a couple of weeks, with yellow to greenish colouration and it may contain mucus/blood or not. It is mostly accompanied with dehydration, and rapid weight loss resulting in high mortality and morbidity (Smitalova *et al.*, 2009). Saif *et al.* in 1980 reported that the disease occurs in piglets of age one to three weeks with a varying peak and depending on the different viral strains it may occur with or without vomiting. The diarrhoea may last from a few days to a few weeks and ideally, there is just a slight weight loss observed in the animals. When pigs 2 -3 weeks old, are infected, the environment becomes heavily infected and younger pigs are easily infected, having about two episodes of the disease in about 3-7 days intervals.

Due to the great economic risk and loss associated with rotavirus infections, there are commercially available veterinary vaccines for the prevention of group A rotaviruses in animals including pigs. These vaccines are given (injection) to the sow in the late stages of gestation so as to induce a passive immunity. Some of these veterinary vaccines are products of Merck and Prosystem (Papp *et al.*, 2013).

2.4.1 Group C Porcine Rotavirus

Morin *et al.* 1990, discovered and identified the group C rotaviruses as the sole cause of enzootic diarrhoea in piglets, hitherto, the group C virus was considered an atypical or a para rotavirus like pathogen which caused a rotavirus like diarrhoea in piglets. The disease is characterised by profuse watery, yellowish diarrhoea with a morbidity rate of

100% observed over 24 h-48 h after birth. In recent times it has been described as an emerging virus infecting humans and animals alike. Reports indicate that it was first found in pigs in the 1980s in the US by Saif and colleagues and subsequently in Europe, Australia and Asia in the same decade. In recent times, they have also been identified in South Africa.

Documented evidence on group C rotavirus is quite limited even though there are reports of sporadic cases found in nurseries and weaning piglets. In their study Kim *et al.* (1999) observed rotavirus C antibodies in older pigs of 8-9 weeks old at very high levels although these groups of rotaviruses had been previously associated solely to suckling and weaning piglets. They also observed clinical signs of profuse watery diarrhoea without vomiting that lasted about two weeks with a high morbidity rate ranging from 60% to 80%.

In humans, the disease aetiology remains unclear, although it has been reported to be milder with less dehydration and vomiting in humans. However, they are increasingly being detected in animals as well. Lee *et al.* in 20

11, identified porcine group C rotavirus in a pig's herd in Korea. Gastroenteric outbreaks in humans by GCRVs is worldwide, thus leading to the recognition of the disease as an important enteric pathogen (Bridger *et al.*, 1986; Szucs *et al.*, 1987; Arista *et al.*, 1990; Caul *et al.*, 1990; Saif and Jiang, 1994; Jiang *et al.*, 1995; Kuzuya *et al.*, 1998; Schnagl *et al.*, 2004; Kuzuya *et al.*, 2005; Rahman *et al.*, 2005; Banyai *et al.*, 2006).

Several studies report 28% to 70% antibody titer prevalence against GCRVs in pigs by the age of 8 weeks and it increases with age up from 79% to 100% in adult pigs (Mathellar *et al.*, 2007; Terret *et al.*, 1987; Saif and Jiang, 1994). Theuns *et al.* in 2016, identified the group C rotavirus in Belgian suckling pigs and they stated that increasingly, knowledge on GARVs is readily available. However, it is not the same with group C rotaviruses. Previous studies of group C rotaviruses identified the virus in diarrheic piglets which showed classic rotavirus pathological lesions in their intestines. They mentioned that group C rotavirus was detected in piglets not older than 3 days in the US, Canada and Mexico. They have associated the group C porcine rotavirus with symptomatic and asymptomatic infections in pigs. Smitalova *et al.* (2009) identified a herd of pigs with asymptomatic infection. The exact aetiology niche, mechanism of infection and clinical symptoms as well as the molecular characteristics of group C rotaviruses remains unclear. This group of rotaviruses has been detected in both outbreaks and sporadic cases, with symptomatic and asymptomatic cases. In Brazil, wild type group C rotaviruses has been identified and characterized and these were found to be more variable than has been proposed and may also contain more than one geno-group based on the VP6 antigen classification (Médici *et al.*, 2011).

In recent times, there has been numerous reports of the Group C virus causing disease in suckling piglets such as outbreak of C rotavirus in suckling piglets in Italy investigated Lorenzetti *et al.* (2014). Suzuki *et al.* in 2014 found porcine rotavirus group C to be responsible for disease in suckling and weaned piglets in Japan and Jeong *et al.* in 2015 also detected group C porcine rotavirus in nursing, weaning, post-weaned, sows and finishing pigs with both symptomatic and asymptomatic infections in Korea. In Ireland,

Collins *et al.* in 2008 detected and observed the infection in 4-5-week-old asymptomatic pig in a study they conducted.

2.5 EMERGENCE OF REASSORTANT ROTAVIRUS STRAIN

It is reported that despite the high load of RV disease in sub-Saharan Africa, because of the range of co circulating diversity strain of the virus there is very limited country specific data on RVA genotypes available and these mostly target genome segments 4 (VP4) and 9 (VP7) (Mwenda *et al.*, 2014; Seheri *et al.*, 2014).

The diversity in RV is engendered by several mechanisms, such as (i) accumulation of point mutations (genetic drift) which causes antigenic changes, (ii) reassortment (genetic shift) which can occur as a result of exchange between two RVA strains (human–human, animal–animal or animal–human strains) leading to viruses with novel genetic and antigenic characteristics, (iii) direct transmission of animal strains into a human host (interspecies transmission) and (iv) gene rearrangement in the coding or noncoding regions, primarily in the non-structural genes (Estes and Kapikian, 2007; Kirkwood, 2010).

Reports from Rahman *et al.* (2007) indicate that rotavirus characterisation has mainly concentrated on the G (VP7) and P (VP4) when genotyping with RT-PCR and Sanger sequencing. Thus, detection of any of the other (9) genome segments would go unnoticed and also the G and P genotyping is not always confirmed by full genome sequencing especially in mixed infections.

Reports indicate that this is due to the probability that some of the genotypes detected by the conventional PCR were missed during sequencing because of the specific priming bias of the sequencing system. They further stated that a strain MRC-DPRU1491 which was genotyped as G1P[4] was later sequenced as G2P[4], P[8] meaning that sequencing picked an extra segment(P8) which is mostly associated with G1 and extra genotypes typical of C1 and M1 suggesting that G1 probably existed in the stool but was missed by sequencing. They also reiterated that, mixed RV infections with distinct genotypes may result in emergence of a new progeny following interspecies transmission and genome segment reassortment. They have reported of the first African G9 human strain which has a genome segment 9 (VP7) in lineage with VI and in close relation with G9 porcine sequences which pertained to lineage III (Page *et al.*, 2010).

Reports also indicate that, an RVA infection varies between geographical areas and from one season to the other. The viruses carry either G1,G2,G3, or G4 and P4 or P[8],and these are the most frequently observed in humans however different investigations have indicated that the G1P8,G2P4,G3P8 and G4P8 are the most common G and P types, (Delorme *et.al* 2001;Esona *et.al*, 2015). Nonetheless, current molecular based typing methods in the past ten years have reported other G and P types in other parts of the world. (Delorm *et.al*,2001). Human viral strains that possess genes commonly found in animal rotaviruses have been isolated from sick children both in developed and under developed countries. G3 strains which are commonly found in humans are also found in cats, dogs, monkeys, pigs, mice, rabbits, and horses. G5 is found in pigs and horses, G6, G8 and G10 are all found in cattle whilst G9 is found in pigs and lambs. All of these have been reported in humans all around the world. Examples of some these occurrence are the G5 strains which were found in Children in Brazil, G6 were found in infected

children in Australia and the G8 were identified in children in Malawi, Nigeria, Kenya, Guinea-Bissau, South Africa, and the United Kingdom (Kelkar *et al.*, 2004). There have also been reports of G9 rotavirus strains found to infect the human population in Brazil, Australia, India, the United States, Bangladesh, Malawi, Italy, France, the Netherlands, and the United Kingdom.

The eventual characterisation of the previously known untypables by routine methods resulted in the identification of unusual strains from Pune (Kelkar *et al.* 2004) and G8 strains from Vellore (Kang *et al.*, 2002). Other P types which were termed untypables such as P11 common in bovine, P[6] common in porcine, P9 common in feline common in porcine and lapine and P19 found in porcine, have all been detected in humans (Desselberger *et al.*, 2001). So many of these strains are possibly of animal origin or animal–human reassortants having one or more genes highly identical to animal rotavirus genes.

In their study of samples (human, porcine and bovine) from sub-Saharan Africa, Nyaga *et al.* (2014), reported of the detection of at least one segment of each sample for which the duplicate sequences always had different genotypes. This suggested that the samples were taken from individuals with mixed rotavirus infections. Their study detected the first porcine (MRC-DPRU1567) and bovine (MRC-DPRU3010) mixed infections and the presence of a unique genome segment of the VP7 whose G9 genotype pertains to the VI lineage and clusters with porcine reference strains. However, the G9 African strains has always been a lineage III. They also isolated RVA segments from humans which had an evolutionary correspondence with porcine, bovine and ovine rota sequences, thus suggesting a recent interspecies transmission and reassortment indicating that multiple

RVA strains are affecting mammalian host in sub-Saharan Africa with unforeseeable disparity in their gene segment combinations.

Kahtoor *et al* 2017, discovered human-like rotavirus C in Indian pigs after a sequence analysis of the VP6 gene from some selected fecal samples positive for porcine rotavirus C strains. Their findings indicate a 93% homology between their selected porcine rotavirus C (PoRVC) to human rotavirus C (HuRVC) which had 12 genotype and of Eurasian origin. In spite of this finding, they realised that the NSP4 and the NSP5 of the same strain were of porcine descent thus suggesting the occurrence of a reassortment due to zoonotic transmission.

2.6 ROTAVIRUS VACCINE:

The first vaccine was derived from a bovine strain from a calf in Nebraska, thus was given the name Nebraska Calf Diarrhoea Virus or NCDV. Trials of the virus in Finland and Venezuela was found to be 80%-100% effective prevention against moderate to severe infections in infants and young children who were orally inoculated with one or two doses of the NCDV (Vesikari, 1998).

The subsequent trial was a simian rotavirus isolates from rhesus monkey called Rhesus rotavirus or R.R.V. This was again successful in Finland and Sweden. However, due to the inconsistency of the viral candidate in it was not pursued. Another strain that was tested in infants and young children was a non-human strain isolated from a calf in Pennsylvania. These were cell cultured at the Wistar Institute, thus derived the term, Wistar calf 3 or WC3 and it offered a hundred percent (100%) protection against moderate to severe infections (Clark *et al.*, 1988).

Later trials of the vaccine in some parts of the USA and Central African Republic were unsuccessful. However, another non-human candidate was tested. Researchers over the years have tried to transform the non-human strains into a more human like strain by retaining the attenuated virulence of the non-human and yet include human rotavirus proteins to elicit a viral neutralisation reaction and to provoke a probable release of protective antibodies. (All trials were done with NCDV, RRV and WC3 offered a level of tolerance). Of the non-human strains, only the RRV is similar to the human G type3.

To maintain the viability and virulent nature of an attenuated non-human rotavirus and at the same time include a more tolerable human rotavirus gene in position to determine the serotypes, it is necessary to comprehend that the virus characteristically is a naked virus with an outer capsid comprising of Viral Proteins 4 &7(VP4&VP7). These VPs are the neutralising antigens that elicit the release of neutralising antibodies during viral infectivity and they determine neutralisation phenotype or serotype (Estes & Cohen, 1989; Barnie *et al.*, 2010).

Once an 11 genomic segmented double stranded RNA is challenged with two different rota strains in the same cell at the same time, the gene segment will reassort itself forming a new virus. Therefore comparatively, it is less cumbersome to produce rotavirus strain that has gene segments from different rotavirus parents (Barnie *et al.*, 2010). However, researchers have come up with rotavirus strains made up of animal rotavirus gene strains and one human serotype determinant gene of human rotavirus strain.

Reports from Hoshino *et.al.* (1993) indicate that researchers from National Institute of Health have formed a reassortant strain obtained from primate gene strain RRV except for the gene encoded VP7 obtained from human rotavirus strains of G types 1,2 and 4 VP7 from RRV is similar to human G3. Hoshino *et.al.* (1993) discovered that the rotavirus virulence is determined by certain four genes even though their single reassortants were not likely to be pathogenic to humans. Trials with RRV(G 3 type) and RRV-Human reassortant G types 1, 2 and 4 was found to be 50% effective against all rotavirus disease and 80% effective against moderate to severe rotavirus infection. Children administered with the first dose of the vaccine experienced episodes of temperatures higher than 38°C fevers, decreased appetite and activity, and irritable however these were not observed during the second and third doses of vaccination.

Other vaccines have been composed such as the WC3 (bovine strain) combination with the human encoding G types 1-4 or P types (Clark *et al.*, 1996). In essence the WC3 – Human strain has minimal side effects as compared to the RRV-Human reassortant strain. A tetravalent (Rotashield, Wyeth-Lederle) option was licensed in 1998 with clinical trials in USA, Finland and Venezuela detecting an effectiveness of 80% -100%. However, the manufacturing company had to withdraw the vaccine because it was realised that it may have contributed to an increased intussusception in children.

Rotarix an attenuated oral monovalent vaccine was approved in 2006 and a pentavalent bovine-human (Rotateq) was also approved for use. These vaccines have proven efficacious and safe with no risk of intussusception in the already vaccinated population in the Industrialised countries and the Latin Americas. They have therefore been introduced as routine vaccine use worldwide. The current vaccines offer a 90%-100%

protection against severe rotavirus infections and a 74%-85% protection against diarrhoea of any severity. In Africa these vaccines are being tried in Ghana and Malawi. India in 2015 unveiled an economically viable vaccine with the support of US National Institute of Health (India Times. Retrieved 25 June 2015).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 CHEMICALS, REAGENTS AND EQUIPMENT

The sources and/or manufacturers of the chemicals, reagents, and equipment used for the study are listed in Appendix I. The various buffers and solutions used were prepared as described in Appendix II.

3.2 STUDY DESIGN

The study was cross-sectional.

3.3 STUDY AREAS

The study was carried out at Akuse in Manya Krobo district including the University of Ghana Farms in Akuse. Akuse is one of the major towns in the Lower Manya Krobo Municipal. The Municipality forms part of the 26 Municipalities and Districts in the Eastern Region of Ghana (ghanadistricts.com, 2006). The Administrative Capital of the District is Odumase. Other major towns in the district include Odumase Township and Kpong in the Lower Manya area.

Agriculture is the backbone of the economy of the District (ghanadistricts.com, 2006). The animals mostly reared in the district include sheep, goats, cattle, poultry, pigs and ducks. Akuse is one of the main livestock rearing areas in the District (ghanadistricts.com, 2006). Because most of these animals are reared for consumption and for sale, most livestock owners living in the compound system prefer rearing them in a kraal (Fig. 3.1) situated in the middle of the house where these animals are provided with water and the necessary feed for subsistence.



Figure 3.1: Typical kraals in a compound house system

Others also practice free range where animals are left to roam indiscriminately to fend for themselves thus in both cases allowing for the scattering of faecal material around the house which poses a potential source of contamination of food in the particular household.

Apart from the above reasons the location was also chosen taking cognisance of the fact that there are many scattered Fulani herdsmen farms in the area, making up several stretches of dotted communities in the bush. These herdsmen live with their various livestock, other animals and family in a makeshift environment where animals and the humans live in very close association. The herdsmen have their kraals in a large opening with their small rooms close by where they live with their families, and kraals filled with the dung of mainly the cattle. However, they also have other small ruminants such as sheep and goats which are not necessarily housed, thus spreading their excreta all around the area. Most of them also keep watch dogs and their main source of water is from borehole/well water for inhabitants and animals alike. On the other hand, in the households at the University farms, farmers who have kraals for their animals, even

though close to their residencies, mostly feed the animals on cut grass provided by the owners. Their main source of water for the animals is pipe borne water.

In the Manya Krobo District the water systems consist mainly of pipe-borne water, boreholes, hand-dug wells and streams. The form of liquid waste disposal in the District is predominantly the household open latrine, especially in the rural areas. (ghanadistricts.com, 2006).

3.4 STUDY POPULATION

Study population included individuals and animals in households where animals (livestock) co-habited with people in the same compound. Communities where people generally dwelt in the outskirts of the townships, almost in the bush with their animals and family were also included. The latter group were targeted due to the living conditions in the bush.

3.5 ETHICAL CONSIDERATION

This study was approved by the Ethics and Protocol Review Committee of the School of Biomedical and Allied Health Sciences, College of Health Sciences, University of Ghana, Korle-bu, Accra. Participants were fully informed about the purpose, procedures, risks, and benefits of participating in this study and those who agreed to participate were recruited after they have signed/thumbprinted an informed consent form (Appendix III). Each participant was assured that their responses would be kept confidential and data collected will be kept only for the purpose of the study.

3.6 SAMPLE COLLECTION

3.6.1 Questionnaire

All members of the households including their animals, both healthy and sick, with or without diarrhoea, were sampled. Prior to sampling, questionnaires (Appendix IV) were administered to farmers of selected households to establish a demographic outlook of each household, the frequency of the incidence of diarrhoea in the household amongst both animals and humans and the seasons of frequent occurrence.

3.6.2 Collection of faecal samples:

Clean, leak-proof, screw capped plastic containers were given to human subjects to collect their stool samples. Animal samples were collected by inserting the index finger in the anal orifice (Fig. 3.2a) to pick faecal samples into clean screw containers.



Figure 3.2a: Collection of animal faecal sample



Figure 3.2b: Human sample collection

Faecal samples obtained from both humans (Fig. 3.2b) and animals were transported on ice to Department of Medical Microbiology of the School of Biomedical and Allied

Health Sciences for processing, stored at -20°C and subsequently sent to the Virology Department of the Noguchi Memorial Institute

3.7 LABORATORY ANALYSIS

3.7.1 Preparation of Faecal Samples

An amount of 0.1 g of faecal extract was added to 1 ml of PBS in a 1.5 ml Eppendorf tube. This was vortexed for 10 seconds and then centrifuged at 12,000 rpm to obtain a supernatant from which RNA was extracted.

3.7.2 Characterization of Rotavirus Strain

The 10% faecal extraction was subjected to polyacrylamide gel electrophoresis procedures (PAGE) to detect the genomic variations of the positive samples to rotavirus strains for subsequent molecular characterisation.

3.7.2.1 dsRNA extraction from stool samples for PAGE analysis

An amount of 200 µl of purified 10% of faecal suspension was mixed with 200 µl of Bender buffer (0.1 M NaCl, 0.2 M sucrose, 0.1 M Tris-HCl pH 7.5, 0.05 M EDTA, pH 9.1, 0.5% SDS) in an Eppendorf tube. The suspension was incubated at 65°C for 30 minutes in a heat block. Then, 60 µl of 8 M KOAc was added, mixed by inverting the tube a couple of times, followed by incubation for 45 minutes on ice. The mixture had to be spun at 1000 rpm for 20 minutes to obtain clarity. Two volumes of cold absolute ethanol were added to the supernatant and the mixture left to stand for 10 minutes. Nucleic acid pelleted by centrifugation at 1000 rpm for 20 minutes, then re-suspended in 200 µl of Tris-EDTA (TE) buffer all at room temperature for 30 minutes. Genomic viral RNA was purified and precipitated with 10 µl of 5 M sodium chloride and cold absolute

ethanol, then incubated at -20°C for an hour. The mixture was spun at 1000 rpm for 20 minutes, and then the pellets dried under vacuum and re-suspended 15 µl of loading buffer.

3.7.2.2 Polyacrylamide gel electrophoresis (PAGE)

PAGE was done for all samples (both animals and humans) in a vertical Hoeffer apparatus. Two gels were prepared (10% resolving gel and 3% stacking gel) and cast between two glass plates (16 x 16 cm) separated by a 1.5 mm spacer. The resolving gel was allowed to polymerize by overlaying with water, which was decanted after polymerization and 3% stacking gel poured on the set up to enhance the resolution of the gel. A sample comb was inserted and the gel allowed to completely polymerize. Extracted double stranded RNA was loaded into different lanes of the gel and electrophoresed for 18-20 hours at 100 V using discontinuous buffer systems. Gels were visualized by silver staining technique as described by Herring *et al.* (1982).

3.7.2.3 Silver nitrate (AgNO₃) staining

The electrophoresed gel was removed from the plates and carefully cut at the bottom right to indicate gel orientation /load. Gels were carefully placed in rubber bowls and fixing solution one added. This was incubated for 30 minutes on an Orbital shaker, subsequently fixing solution one was drained off and an already prepared silver nitrate solution was added and gels incubated on the orbital shaker for 30 minutes. The silver nitrate solution was drained off and gel was washed twice with distilled water, 2 minutes per wash. Then 50ml of developing solution was added and gel agitated for 30 minutes to remove all residual black precipitates. Once it was drained a second developing solution was added and incubated for 5 minutes, till electrophoresed RNA bands were

visible. The developing solution was drained and a stopping solution was added to avoid further colouration of the bands. Gels were rinsed in distilled water and subsequently observed under light and pictures taken for strain identification and characterization.

3.7.3 Extraction of Viral RNA for VP7 and VP4 Genotyping

Positive samples (double stranded RNA) both from humans and livestock (cattle, sheep, pigs and goats) for PAGE were selected for a second extraction by the standard phenol-chloroform extraction procedure. The dsRNAs were then purified with RNaid kit (Bio 101, Carlsbad, USA) following the manufacturer's protocol.

Fifty microliters of 1 M sodium acetate containing sodium dodecyl sulfate (SDS) (pH 5.0) was added to 500µl of 10% of faecal suspension in an Eppendorf tube. This was vortexed for 10 seconds and incubated at 37°C (water bath) for 15 minutes. Five hundred microliters of phenol chloroform (1:1) was added to the tube and vortexed for 1 minute. Subsequently they were incubated in water bath at 56°C for 15 minutes, centrifuged at 10,000 rpm for 3 minutes. The supernatant was removed into a new Eppendorf tubes and the pellet discarded.

Another 250µl of phenol/chloroform (1:1) was added into the new eppendorf tubes vortexed for 1 minute and incubated in a water bath for 56°C for 15 minutes and subsequently vortexed for 1 minute and centrifuged for 10,000 rpm for 3 minutes, the supernatants were removed into new eppendorf tubes and pellets discarded. Five hundred microliters of GITC was added to the new eppendorf tubes, vortexed and centrifuged at 12,000 rpm for 5 minutes. RNaid matrix (supplied with the RNA extraction kit) was vortexed and 10 µl added to each eppendorf tubes. The eppendorf

tubes were vortex for 30seconds, incubated on a rocker at room temperature for 15minutes and centrifuged at 12,000 rmp at 10 seconds.Supernatants were discarded and four hundred microliters of RNaid wash (in extraction kit) was added to the pellets and resuspended gently with a pipette, centrifuged at 12,000 rmp for 30 seconds.Again supernatants were discarded and 100ul of the RNaid wash was added to the pellets, resuspended gently with a pipette and centrifuged for minute at 12,000 rmp.

Supernatants were discarded and pellets resuspended with DEPC treated water (in extraction Kit) and incubated at 65°C for 10minutes to elute the RNA formed beads. The eppendorf tubes were were centrifuged for 3minutes at 12,000rpm, supernatant containing extracted RNA were transferred into new sterile Eppendorf tubes and stored at -20°C for further RT-PCR reactions.

3.7.4 VP7 and VP4 Genotyping in Humans

The VP7 and VP4 genotype was done as described by Gouvea *et al.* (1990) and Gentsch *et al.* (1992), respectively, using the primers shown in Tables 3.1 and 3.2. The three-step method involved reverse transcription (RT), amplification of the transcribed gene (PCR) and multiplex PCR to determine the genotypes using specific primers.

3.7.4.1 VP7 genotyping

3.7.4.1.1 Reverse transcription and PCR amplification of the transcribed gene

The purified ds RNA was reverse transcribed as described by (Gouvea *et al.*, 1990) using the specific primers sBeg9/ End9. The ds-RNA was denatured by heating at 94°C for 5 minutes, cooled immediately in an ice bath for 2 minutes. The denatured strands were then reversely transcribed by the addition of 3.2ul of the master mix (all four

DNTPs, 0.4 µl of avian myeloblastosis (AMV) reverse transcriptase and 2.0 ul of 5X AMV buffer) and incubated for 20 minutes 42°C in a water bath. The cDNA was then amplified by PCR in a 40 ul reaction mixture containing (0.25 ul of 10 mM dATP, 0.25 ul of 10 mM dGTP, 0.25 ul 10 mM dTTP, 10 ul of 5X Taq buffer, 25.55 ul of double distilled water and 0.25 ul Taq polymerase) prior to use. Thirty-five reactions cycles of PCR (1 minute denaturing at 94°C, 2 minutes annealing at 42°C, 3 minutes extension cycle at 72°C) and final extension at 72°C for 7 minutes was performed in a Gene AMP PCR Primus 25 system machine. The amplified samples were loaded onto agarose gel containing 4 ug/ml of ethidium, bromide and electrophoresed in tris-acetate EDTA (TAE) buffer at 100 V for 30-45 minutes and the size of the amplified products visualized. Negative and positive controls were included in each PCR reaction.

Table 3.1: Oligonucleotide primers for G genotyping (Gouvea *et al.* 1990).

Primer	Sequence (5'-3')	Position (nt)	Strain (genotype)
sBeg9	GGCTTTAAAAGAGAGAATTC	1-21	Group A
Beg9	GGCTTTAAAAGAGAGAATTTCCGTCTGG	1-28	Group A
End9	GGTCACATCATAACAATTCTAATCTAAG	1062-1036	Group A
aAT8	GTCACACCATTTGTAAATTCG	178-198	69M (G8)
aBT1	CAAGTACTCAAATCAATGATGG	314-335	Wa (G1)
aCT2	CAATGATATTAACACATTTTCTGTG	411-435	DS-1 (G2)
aDT4	CGTTTCTGGTGAGGAGTTG	480-498	ST-3 (G4)
aET3	CGTTTGAAGAAGTTGCAACAG	689-709	P (G3)
aFT9	CTAGATGTAACACTACAACACTAC	757-776	W161 (G9)
RVG9	GGTCACATCATAACAATTCT	1062-1044	Group A

Table 3.2: Oligonucleotide primers for P genotyping (Gentsch *et al.*, 1992)

Primer	Sequence (5'-3')	Position (nt)	Strain (genotype)
1T-1	ACTTGGATAACGTGC	339-356	KU (P8)
2T-1	CTATTGTTAGAGGTTAGAGTC	474-494	RV5 (P4)
3T-1	TGTTGATTAGTTGGATTCAA	259-278	1076 (P6)
4T-1	TGAGACATGCAATTGGAC	385-402	K8 (P9)
5T-1	ATCATAGTTAGTAGTCGG	575-594	69M (P10)
Con 3	TGGCTTCGCCATTTTATAGACA	11-32	Group A
Con 2	ATTTCGGACCATTTATAACC	868-887	Group A

3.7.4.1.2 Multiplex PCR to determine VP7 genotypes

The rotavirus strain was determined in a second-round PCR. Two microliters (2 ul) of the first-round reaction products were used in a master mix containing each serotype-specific primers (aBT-1, aCT-2, aET-3, aDT-4, aAT-8, aFT-9, G10, G12, RVG9), 0.2 ul of 10 mM dATP, 0.25 ul of 10 mM dCTP, 0.25 ul of 10 mM dGTP, 0.25 ul of 10 mM dTTP, 10 ul of 10X Taq buffer, 27.75 ul of ddH₂O and 0.3 ul of *Taq* polymerase. PCR was done as described by Gouvea *et.al.* (1990) and the amplicons analysed in 2% agarose gels. Genotypes were determined from the sizes displayed on the gels.

3.7.4.2 VP4 genotyping

3.7.4.2.1 Reverse transcription and PCR amplification of rotavirus VP4 gene

P- Typing RT-PCR was performed using the primers Con 2 and Con3 (Gentsch *et al.*, 1992) using the protocol described earlier. Multiplex PCR to determine VP4 genotypes was carried out in a second-round amplification using consensus primers Con 3 and a

cocktail of animal genotype-specific primers (IT-1D, 2T-1, 3T-1, 4T-1 and 5T-1) as described by Genstch *et al.* (1992).

3.7.5 VP7 and VP4 Genotyping in Animals

VP7 genotyping was determined in a second-round multiplex PCR as described above for humans but using the serotype-specific primers (aFT-5, aDT-6, aHT-8, aET-10, aBT-11) (Genstch *et al.*, 1992). Amplicon were analysed in ethidium bromide stained 2% agarose gel. Genotypes were determined from the sizes of amplicons displayed on the gels.

VP4 genotyping by P-typing RT-PCR was performed using the same PCR protocols used for humans. The rotavirus strain was determined in a second-round multiplex PCR as described above for humans but using the primers Con3 and a cocktail of animal genotype-specific primers (pGott, pOsu, pUk, pB223, pNCDV) (Gentsch *et al.*, 1992). Amplicons were analysed in ethidium bromide stained 2% agarose gels. Genotypes were determined from the sizes of amplicons displayed on the gels.

CHAPTER FOUR

4.0 RESULTS

4.1 DEMOGRAPHIC CHARACTERISTICS OF STUDY HOUSEHOLDS

A total of 22 households with of a total of 173 persons, 71 (49%) males and 102 (51%) females, were surveyed. The age range was 3-54 years. The number of persons in the household ranged from 4 – 16 (Fig. 4.1). Majority (50%) of the households lived in isolated cottages (normadic) (Fig. 4.2).

A total of 228 animals were sampled from the 22 households. Goats (37%) were the most animals sampled and pigs (17%) the least (Fig. 4.3a). The distribution of animals per household varied (Fig. 4.3b). Some households reared only one species of animal while others had a mixture of species.

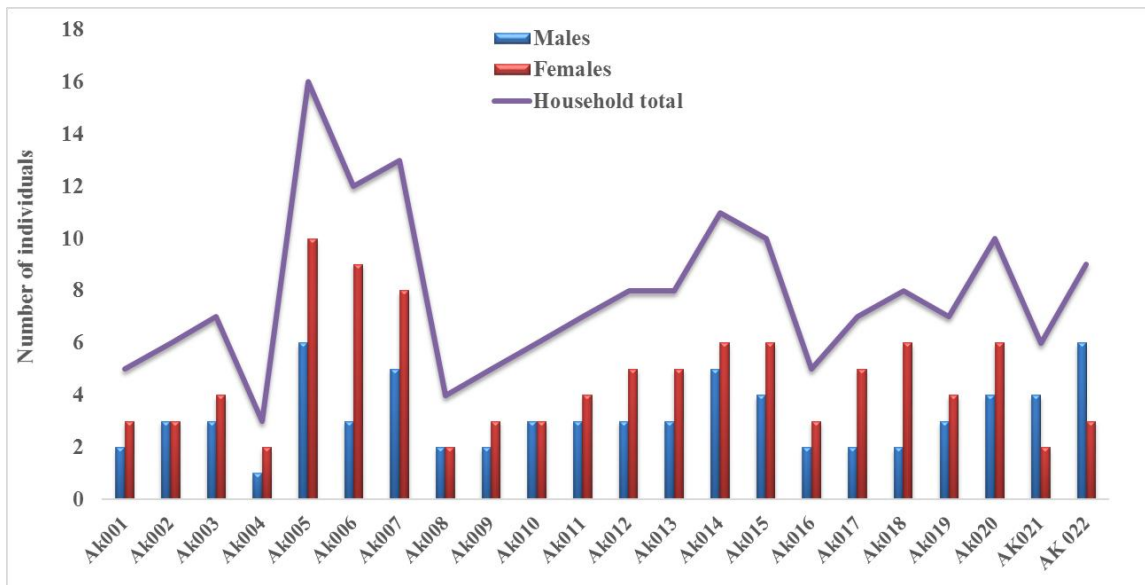


Figure 4.1: Household populations

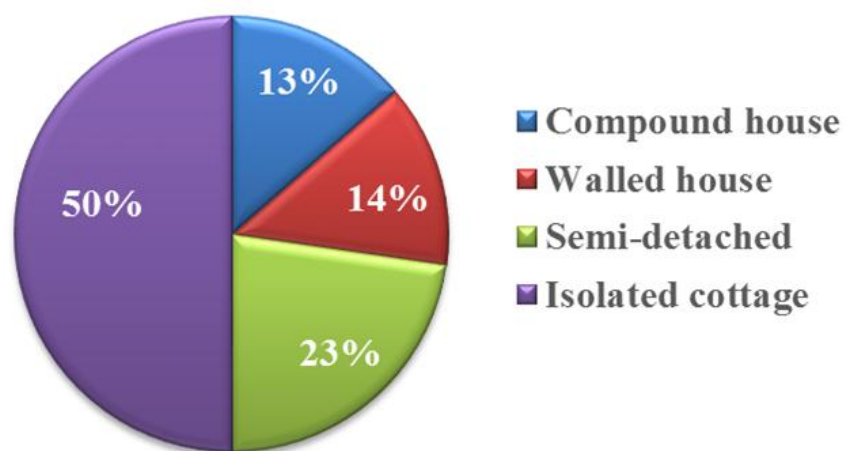


Figure 4.2: Housing types

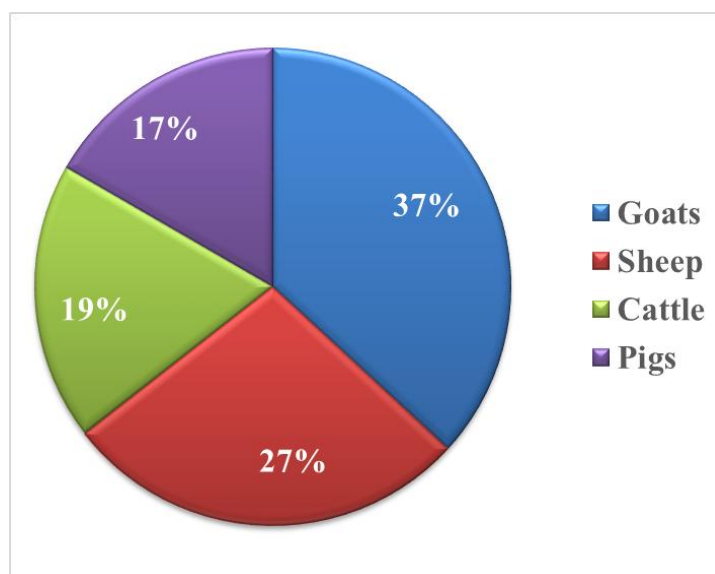


Figure 4.3a: Type of animals sampled

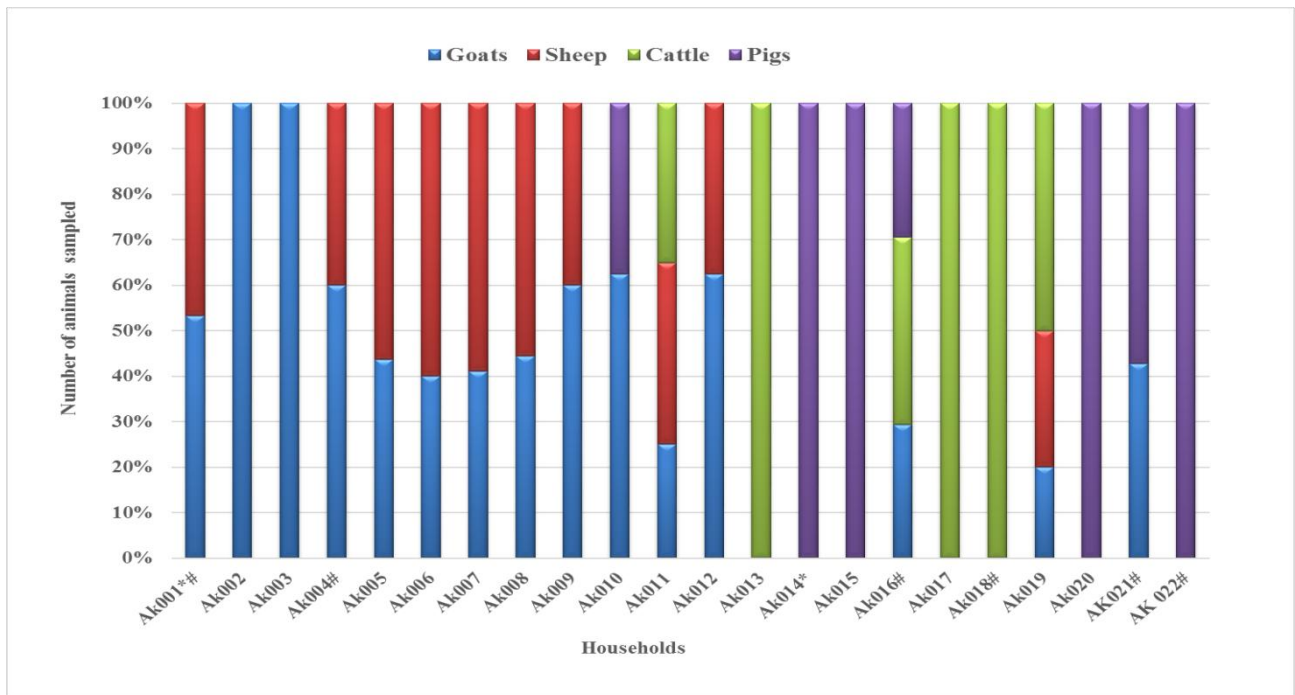


Figure 4.3b: Distribution of animals sampled per household

4.2 FREQUENCY OF HOSPITAL VISITATIONS AND COMMON HEALTH COMPLAINTS OF HOUSEHOLDS

Figure 4.4a shows the frequency of hospital visitation by the households. The most frequent Hospital visitations were thrice a year with 27% and twice a year 23%.

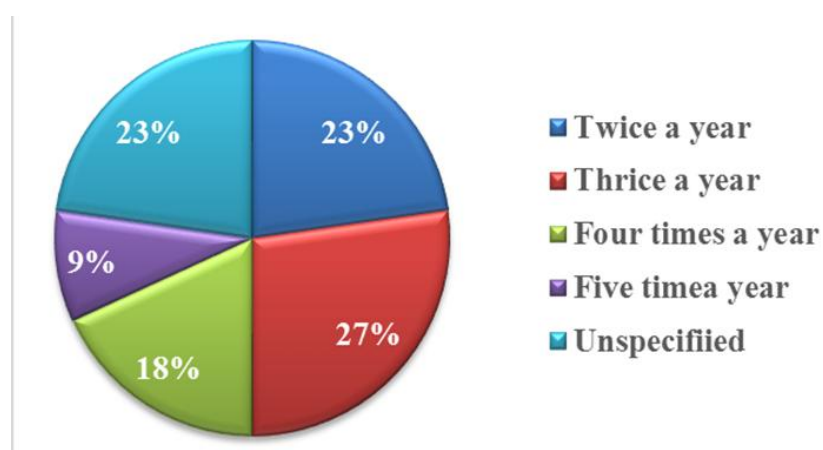


Figure 4.4a: Frequency of hospital visitations

Fever (73%), stomach ache (57%) and diarrhoea (45%) were the most common health complaints from the households. Backpain and toothache (5% each) were the least complaints (Fig. 4.4b).

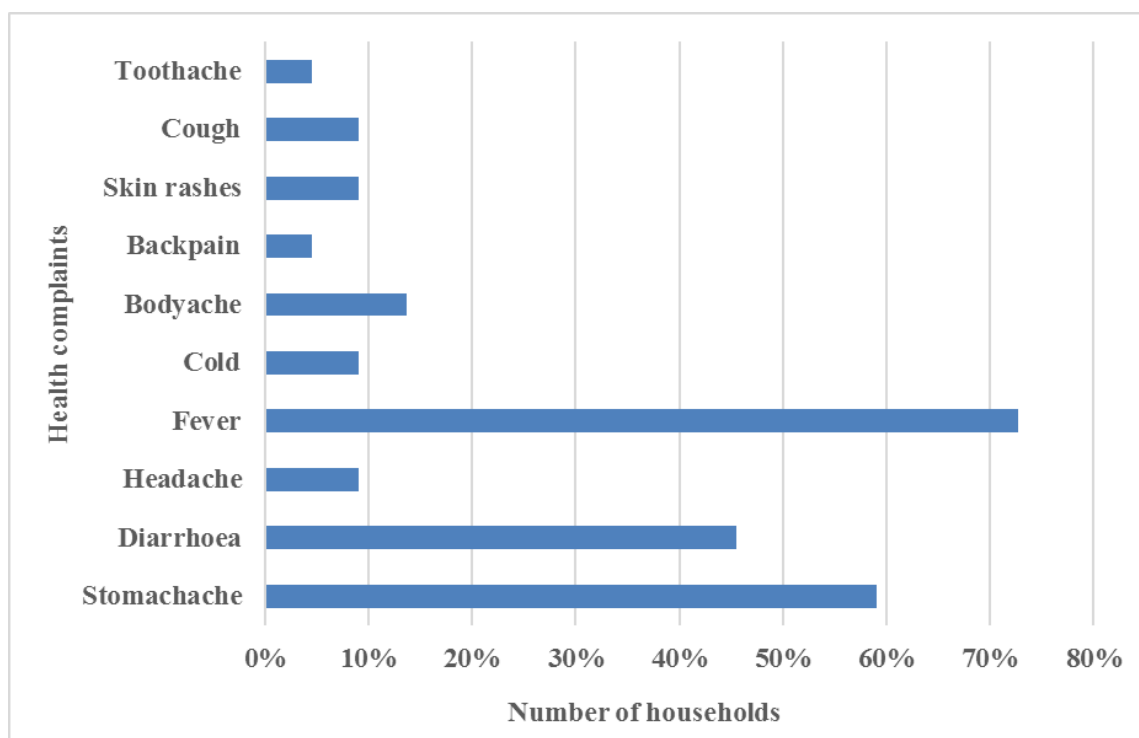


Figure 4.4b: Common health complaints from the households

4.3 REARING METHODS AND MAINTENANCE OF ANIMALS

Data on rearing method and maintenance of animal were obtained from all the households (N = 22). Three different rearing methods were practiced with semi-intensive method (75%) the highest rearing method practiced (Fig. 4.5). Moderate level of hygiene (59.09%) in the pens/kraals was mostly observed (Fig. 4.6). Frequency of cleaning of pens/kraals varied with twice per week (45.45%) being the most practiced (Fig. 4.7). Water sources were either well only (45%), pipe borne only (23%) or both (32%).

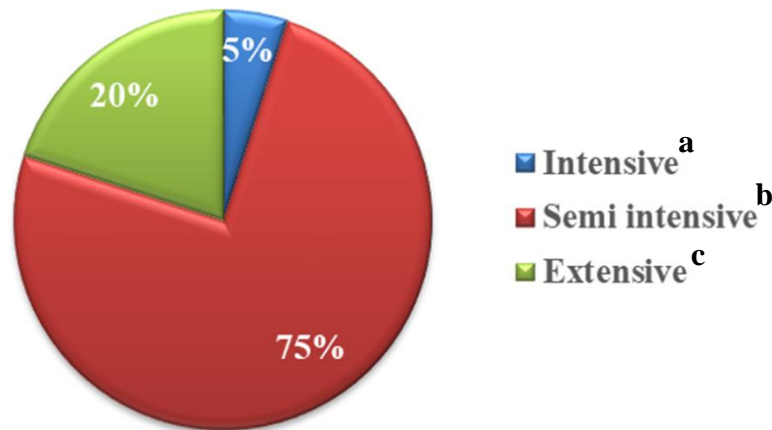


Figure 4.5: Rearing methods used.

^aThis method of rearing is capital intensive, concentrates the animals in a defined housing space, provides them with required living conditions and feeds them with fodder, crops, grasses etc. at stipulated amounts within a period of time to achieve a maximum growth. ^bIs an intermediate system between the intensive and extensive systems where flocks have limited grazing. ^cUses small inputs of labor and capital, animals are usually released for free range grazing during the day, whilst some form of grass and water is provided for them once they return in the evening. They may or not have any form of housing.

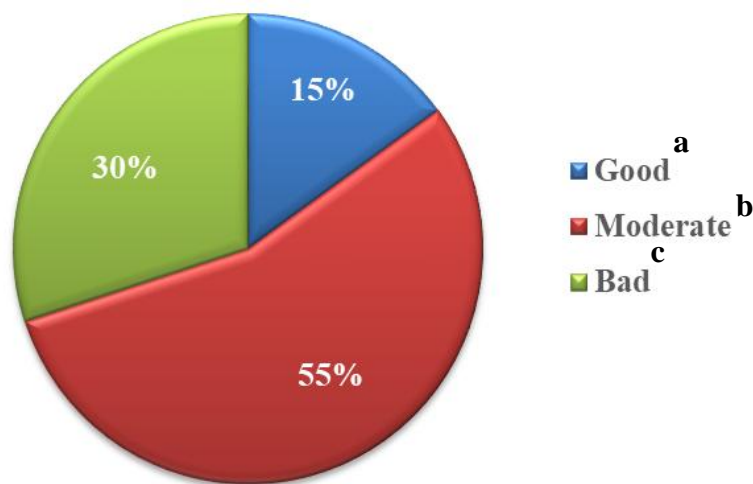


Figure 4.6: Level of hygiene in pens/kraals

^aLess than 10% fecal materials present, no spillage of water, proper bedding, presence of wallows, sun radiation covers half of the pen. ^bMore than 50% of floor covered with fecal material, spots of spilled water (damp floors), and little aeration. ^cMore than 80% floor space covered in fecal material, absence of protective roofing.

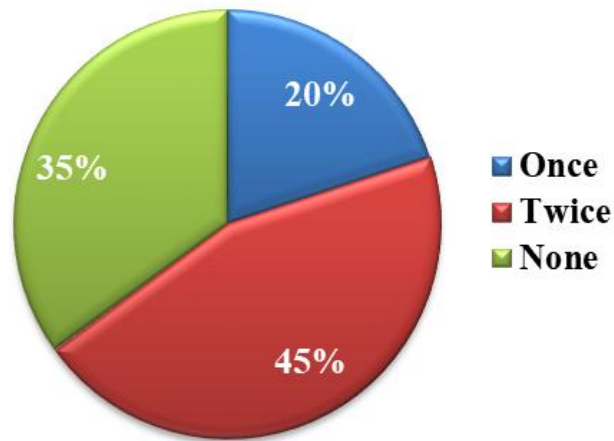


Figure 4.7: Frequency of pen/kraal cleaning

4.4 COMMON HEALTH CONDITIONS REPORTED AND TREATMENT OF SICK ANIMALS BY HOUSEHOLDS

Diarrhoea (64%) and ectoparasites (27%) were the most common health conditions reported (Fig. 4.8). Stomach upset (5%) was the least complaint reported. Treatment of a sick animal (Fig. 4.9) was done either by a household member or a Veterinary officer or both. Treatment by a household or a Veterinary officer (45%) was the highest form of treatment. However, for one household no one was (5%) responsible for treating the sick animals

4.5 FREQUENCY OF HOSPITAL VISITATIONS AND COMMON HEALTH COMPLAINTS (HUMANS)

Frequency of hospital visitations and the common health complaints reported are shown in table 4.1 and Fig. 4.10, respectively. Visitations of thrice a year (27.3%) and twice a year (22.7%) were the most frequent. Fever (73%), stomach ache (59%) and diarrhoea (45%) were the most common health complaints from the households. Backpain and toothache (5% each) were the least complaints.

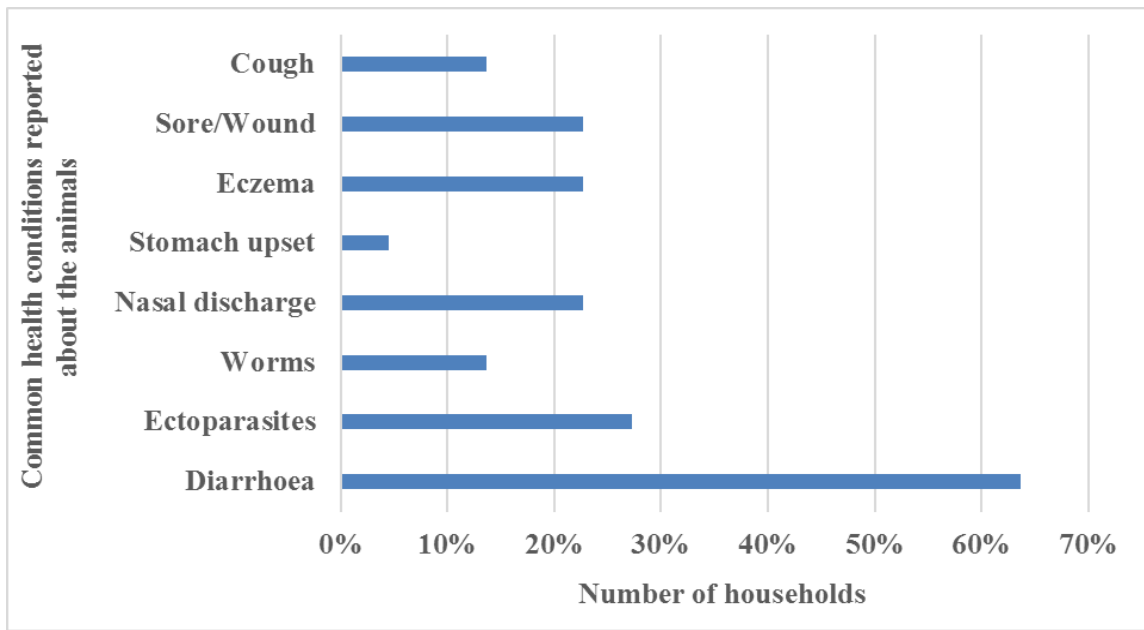


Figure 4.8: Common health conditions reported about the animals by the households

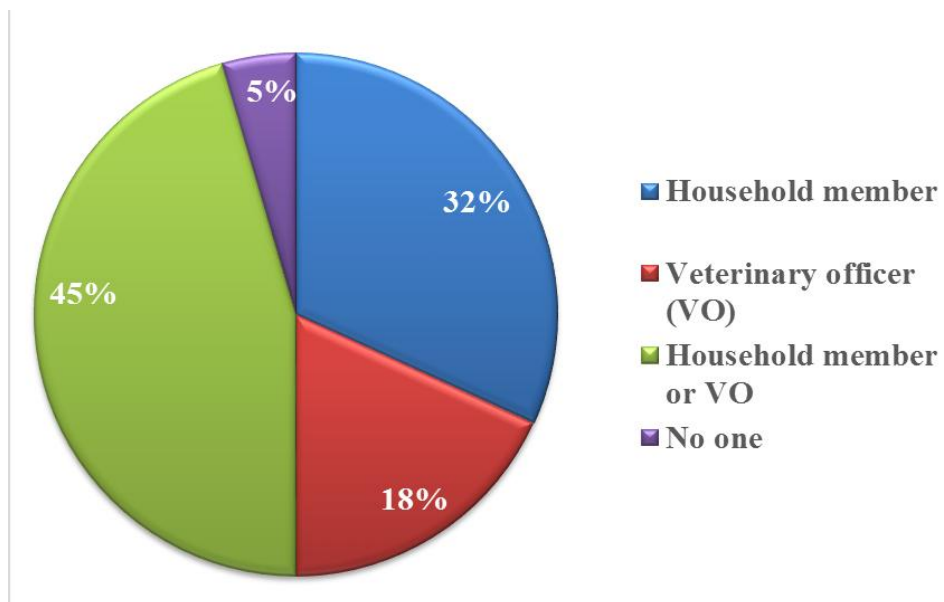


Figure 4.9: Treatment of sick animals

Table 4.1: Frequency of hospital visitation (N=22)

Parameter	Variable	N (%)
Frequency of hospital visitation	Twice a year	5 (22.7)
	Thrice a year	6 (27.3)
	Four times a year	4 (18.2)
	Five times a year	2 (9.1)
	Unspecified	5 (22.7)

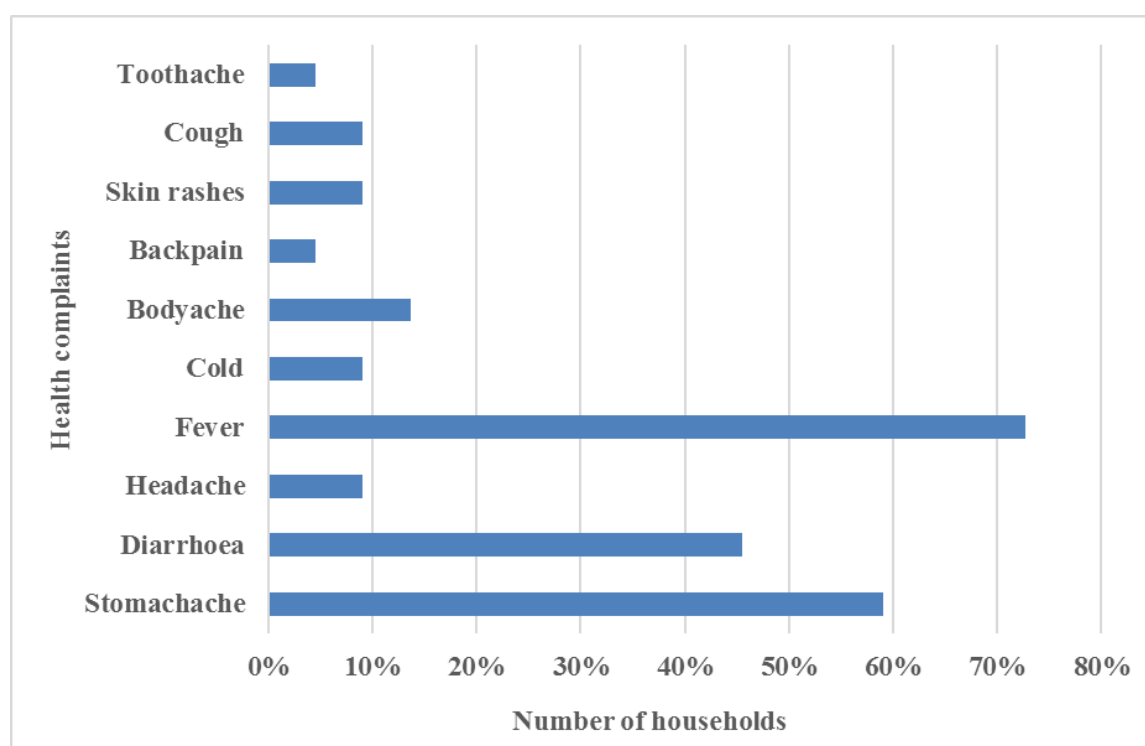


Figure 4.10: Common health complaints from the households

4.6 MOLECULAR ANALYSIS

4.6.1 Extracted RNA Concentration:

The RNA concentration from the extracted samples ranged from 192.85-208.65 ng/ul.

4.6.2 Characterisation of rotaviruses by polyacrylamide gel electrophoresis (PAGE).

Three rotavirus electropherotypes (A, B and C) were detected in animal and human samples during the study period. Electrophoresis patterns for group A, group B, and group C double stranded RNA rotaviruses were observed (Fig. 4.11). Rotavirus was detected in 6 animals from different households. Rotavirus A was detected (Fig. 4.12i) in 6 individuals (3.4%) from 6 different households. Rotavirus B was detected (Figs. 4.12ii) in 2 sheep in another household. Rotavirus C was detected (Figs. 4.12iii) in 4 pigs from one household. One household had Rotavirus A infections in human and Rotavirus B in animals.

4.6.3 RT-PCR and genotyping: VP7

4.6.3.1 Human rotavirus VP7 genotype analysis

Products obtained from the first-round amplification were used for the next 2 to genotype for viral strain. Reverse transcription and PCR amplification (first amplification) of rotavirus dsRNAs with primers Beg9/End9 yielded the DNA fragments of the predicted size (1062 bp) [Figs. 4.13]. VP7 genotyping yielded two genotypes, G8 and G10 (Fig. 4.14), at low frequencies of 3 and 4%, respectively.

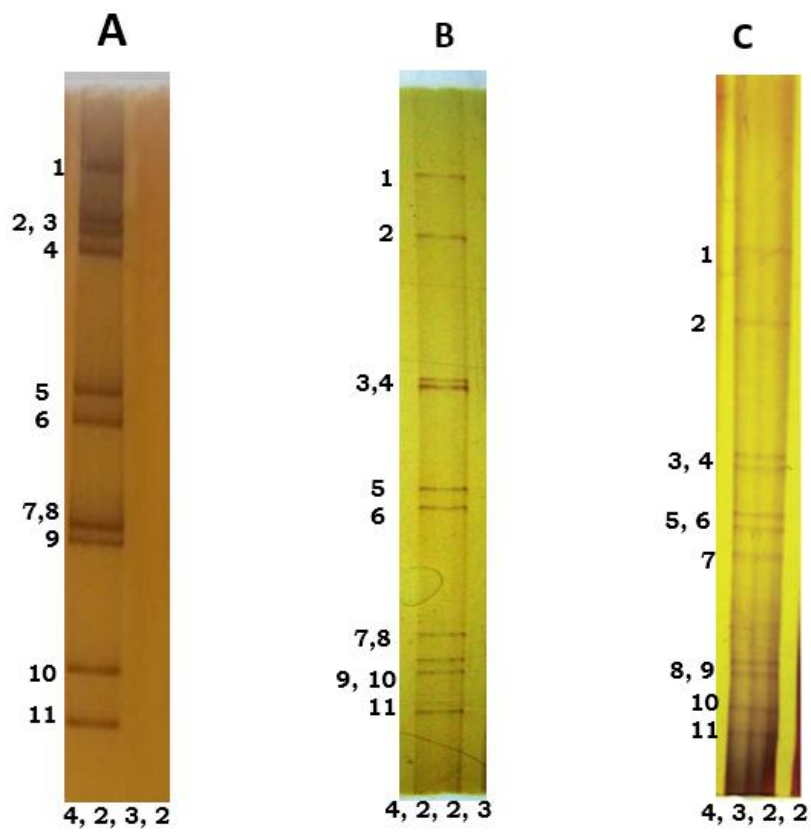


Figure 4.11: Representative RNA electropherotypes of the rotaviruses observed

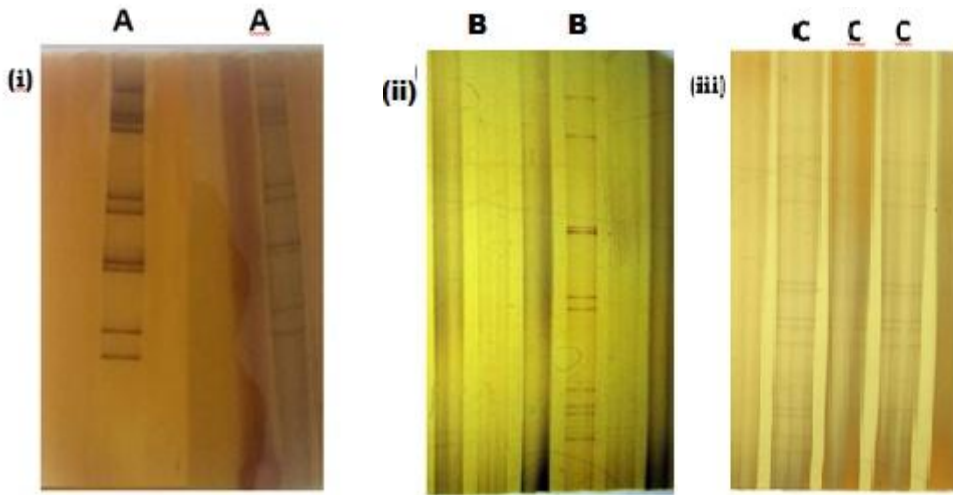


Figure 4.12: Rotavirus RNA migration patterns (electropherotypes) in some of the study samples using polyacrylamide gel electrophoresis (PAGE). (i) Human samples. (ii) and (iii) animal samples

4.6.4 RT-PCR and genotyping: VP4

No amplification products were obtained using primers Con3 and a cocktail of animal genotype-specific primers (pGott, pOsu, pUk, pB223, pNCDV) for VP4 genotyping.

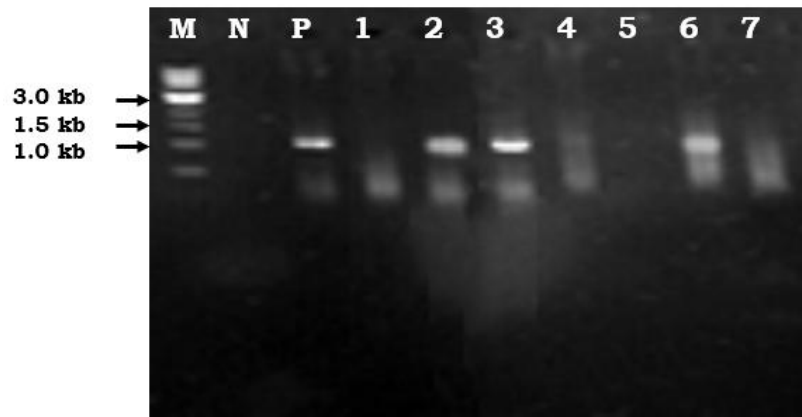


Figure 4.13: Ethidium bromide-stained 1.0% agarose gel electrophoregram of amplified entire VP7 gene using primers End9/sBeg9 or End9/Beg9.

Lane M = 1 Kb DNA ladder (NEB); Lane N = negative control; Lane P = positive control; Lanes 1 = negative human sample; Lanes 2 - 4 = positive human samples; Lane 5 = blank well; Lanes 6 & 7 = positive animal samples

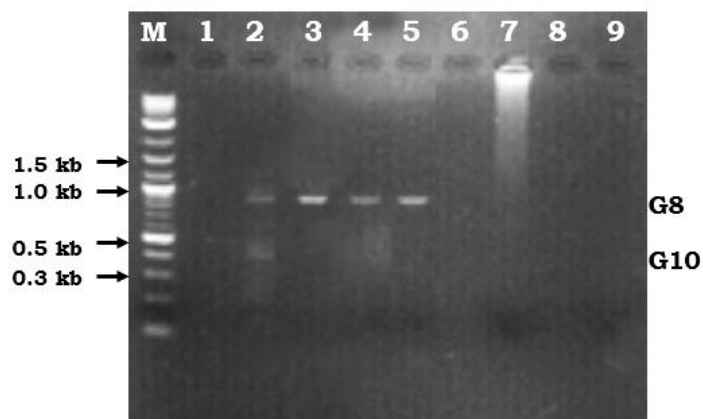


Figure 4.14: Ethidium bromide-stained 1.0% agarose gel electrophoregram of amplified RV genotypes using a cocktail of primers

Lane M = 2-log DNA ladder (NEB); Lanes 1 = negative control; Lanes 2 - 4 = positive human samples; Lane 6 = blank well; Lanes 6 & 7 = negative animal samples

4.7 ROTAVIRUS VP7 GENOTYPES WITH DEMOGRAPHIC INFORMATION

Table 4.2 shows rotavirus VP7 genotypes with demographic information. VP7 genotypes were found where the rearing method was either intensive or semi-intensive. In addition, animal treatments in these cases were mainly by self-treatment (3 out of 4). The type of housing was mainly semi-detached.

Table 4.2: Rotavirus VP7 genotypes with demographic information

Household ¹	Type of Housing	Rearing Method ¹	Animal Treatment	Type of animal	Characterisation (PAGE)	VP7 Genotypes
Ak001*#	Semi-detached	Semi-intensive	Self-treatment	Sheep	A [#] , B*	G8 [#] & G10 [#]
Ak004#	Semi-detached	Semi-intensive	Self-treatment	-	A	
Ak014*	Isolated cottage	Intensive	Self-treatment	Pigs	C	G8
Ak016#	Isolated cottage (Normadic)	Semi-intensive	Self-treatment	-	A	G8
Ak018#	Isolated cottage (Normadic)	Extensive	Veterinary Officer/ Self-treatment	-	A	
AK021#	Semi-detached	Semi-intensive/Extensive	Veterinary Officer/ Self-treatment	-	A	
AK 022#	Isolated cottage (Normadic)	Intensive	Veterinary Officer/ Self-treatment	-	A	G8

[#]Human, *Animal

4.8 WATER SOURCE AND COMMON HEALTH COMPLAINTS OF HOUSEHOLDS POSITIVE FOR ROTAVIRUS

Table 4.3 shows the waater source and common health complaints of households positive for rotavirus. VP7 genotypes were found mostly (3 out of 4) in where a well was the water source. Diarrhoea was the most commom health complanint for both human and animals.

Table 4.3: Water source and common health complaints of households positive for rotavirus

Household ¹	Water source	Common health complaints	Common health conditions reported about the animals	Characterisation (PAGE)	VP7 Genotypes
Ak001*#	Pipe borne, Well	Stomach ache, malaria, diarrhoea	Diarrhoea, skin condition	A [#] , B*	G8 [#] & G10 [#]
Ak004#	Pipe borne	Fever, Body ache	NA	A	
Ak014*	Well	NA ²	Diarrhoea, sores	C	G8
Ak016#	Well	Malaria, diarrhoea, fever	NA	A	G8
Ak018#	Well	Fatigue, malaria, stomach upset	NA	A	
AK021#	Pipe borne	Stomach ache, diarrhoea	NA	A	
AK 022#	Well	Skin rashes, diarrhoea	NA	A	G8

[#]Human, *Animal

CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSIONS

5.1 DISCUSSION

Rotaviruses are considered to be the most common cause of gastroenteritis in young children and young animals worldwide. The main objective of this study was to characterize rotavirus isolates from humans and animals living in close association in communities in and around Akuse, and to determine genotypic variations in the isolates obtained.

The Akuse community in the Manya Krobo district is an agricultural community which practices animal exploitation extensively with isolated Fulani herdsman settlements. Most of these peasant farmers live in close proximity with their livestock and such conditions reportedly foster interspecies transmission of rotaviruses resulting in the generation of novel reassortant strains globally.

Zoonotic transmission of animal rotavirus strain to humans is possible through indirect contact such as contaminated surfaces due to the ability of the virus to remain viable on fomites, environment, and non-living surfaces for several days when dried, posing a strong potential for spread. (Ansari *et al.*,1991; Haffejee, 1995; Torok *et al.* 1997), (Cited by Wandera *et al.* 2012). Contaminated food and water sources in communities whereby animals have direct access to the water source just as the humans also contribute to indirect transmission. In such situations, faecal materials as well as nasal discharges are spread in the household setting and in the water source, making it ideal to promulgate the easy spread of the virus to and from animals/ humans.

This phenomenon allows for easy zoonotic transmission of interspecies reassortants especially in unsuspecting rural settings like the study site (Akuse) where humans and animals live in close association. Amoah-Barnie *et al.* (2010) reported that close co-living of humans and animals may have resulted in interspecies infections due to recombinants from human and animal variants in some four communities in Accra, Ghana. Reports from Gentsch *et al.*, (2005) indicate that zoonotic transmission of rotavirus strains are capable of causing both asymptomatic, mild to severe diarrhoea infections in humans. It has been confirmed by Desselberger *et al.* (2000) that human group A rotavirus strains possess certain genes that are mostly found in animal rotaviruses which have been isolated from infected children in both developed and developing countries. Enweruno-Laryea *et al.*, (2014) reported animal genotype and reassorted variants in pediatric diarrheal cases at the Korlebu Teaching Hospital.

In this study, different RV electropherotypes were detected in the human and animal stool samples as per households analysed. RVA was the major precursor of infection identified in the human stool samples, with all 6 positives exhibiting the typical 4-2-3-2 migratory pattern of the rotavirus A and a prevalence rate of 27.3% at the household level, lower than the 45.7% reported by Barnie *et al.* (2010) obtained from some communities in Accra. Some exhibited long electropherotypes while others were short. Reports indicate that RVA causes more than 90% of RV infections in humans (Kapikian & Shope, 1996; 2007) causing acute infectious diarrhoea in children and the young of animals. RVA is said to be a serious public health problem worldwide (Estes *et al.*, 2007) especially in developing country like Ghana where vaccination programmes focuses solely on human cases and surveillance activities in the country are concentrated

in the Northern part of Ghana where climatic conditions are markedly different from Southern Ghana (Damanka *et.al*, 2003).

According to Papp *et.al*. (2014) methods for nucleotide-based sequencing and genotyping has revealed that RVA strains tend to cross host species barriers resulting in the introduction of heterologous strains in new ecological environments by direct interspecies transmission or reassortment with an RV of the same functional strain. Many epidemiological surveillance world-wide have reported an increase in the diversity of co-circulating rotavirus strain in humans and some attribute this to the use of additional analysis and the type of primer sets being used (Esona *et al.*, 2010). Then again, the complex diversity of rotavirus also progresses through point mutations (genetic drifts or genetic shift) through re-assortment of genes especially during mixed or dual infection of a single host.

In this study, Rotavirus B in two sheep was detected by PAGE with the typical electrophoretic 4-2-2-3 pattern. In the same household a human rotavirus A was detected for which genotypical analysis identified a mixed infection of G8 and G10 in the human positive of that household with a 14.3% prevalence at household level. The G8 and G10 are generally detected in calves or as bovine genotypes, but in recent times these constitutes a significant proportion of HRV especially in Africa (Steel *et al.*, 2003).

In research works done elsewhere, out of the 19 G genotypes that have been detected in humans, type G1-G4 are the most widespread, for which vaccines have been designed and developed. G8 and G10 do not form part of the rotavirus vaccine combinations. This creates the likelihood that RV vaccines with genomes containing a P or G genotype may

not effectively control an infection caused by mixed strains (McDonald *et al.*, 2009) such as a G8 & G10 combination. The G10 which is frequently detected in calves have also been detected in Brazil (Santos *et.al*, 1998), Paraguay (Coluchi *et. al*, 2002), India (Gomara *et .al*, 2004) and Ghana (G.E.A., Unpublished data, Cited by Esona *et.al*, 2010).

Furthermore, in this study genotyping resulted in genotype G8 for all the human positives. While G8 was found at relatively high frequency of 4 (57.1%) in the different households, G10 was at a frequency of 1 (14.3%) in a mixed infection with a G8 in a rotavirus A positive human stool sample both from the same household where rotavirus B was detected in sheep. These results provide evidence of possible interspecies transmission of rotaviruses within some of the households in Akuse where humans and animals are closely associated. Both detected multiple or dual infections and the other positives found in the households were P genotype non typeable by the set of primers used in this study. The frequency of typeability of the G8 was 4 (57.1%) while the frequency for the dual infection was 1 (14.3%) lower than the 38% reported by Barnie *et.al.* (2010) and the 34.4% reported by Gladstone *et.al.* (2008). The sample in lane 1 characteristically shows duplicate but different genotype segments for the same sample, inferring the detection of a mixed infection with two genotypes, G8 and G10 in the same sample.

In their study of samples from human, porcine, bovine from Sub Sahara Africa, Nyaga *et al.* (2014), reported the detection in their samples of at least one segment which had duplicate sequences of different genotypes, thus suggesting that the sample was taken from subjects with mixed rotavirus infections. In their study, Kelkar *et.al.* (2004)

reported that the eventual characterisation of the previously known untypables by routine methods resulted in the identification of unusual strains from Pune (Kelkar *et al.* 2004) and G8 strains from Vellore (Kang *et al.*, 2002). Other P types which were termed untypables such as P [11] common in bovine, P [6] common in porcine, P [9] common in feline common in porcine and lapine and P[19] found in porcine, have all been detected in humans (Desselberger *et al.*, 2001). So many of these strains are possibly of animal origin or animal–human reassortants having one or more genes highly identical to animal rotavirus genes.

In ruminants, most commonly detected of the RV strains belong to RVA strains, but in some settings RVB and RVC are mostly found and implicated in severe diarrhoea, particularly in young lambs and goats (Fitzgerald *et al.*, 1994; Galindo-Cardiel *et al.*, 2011). Ovine RVs belong to either RVA or RVB, however the epidemiology of lamb RVs is still largely unknown (Schoenian *et al.*, 2012).

Several studies have reported RVB detection in 16–100% of the examined stool samples from both diarrheic and non-diarrheic lambs (Theil *et al.*, 2014). In this study RVB was lower at 1 (14.3%) in a non asymptomatic sheep from the same household where RVA was detected in humans as well as G8 and G10 genotypes. RVB in sheep has been known to cause high morbidity of 75–100% during outbreaks of neonatal diarrhea in lambs and remarkable mortality has also been reported. RVB has also been found in humans during epic out breaks with high morbidity, even though the distribution is limited; China, Bangladesh and India (Hung *et.al*, 1998; Sanekata *et.al*, 2003). An epidemiological surveillance in Bangladesh reported that Group B rotavirus is considered to be virulent in both adults and children, and the virus may be a serious

diarrheal agent in Bangladesh. In their survey, 12/220 adults and 2/67 children were diagnosed with severe diarrhoea in Bangladesh (Sanekata *et al.*, 2003). The epidemiological significance of the detection of RVs for domestic animals (livestock) in our human subjects in this study can not be overly emphasized.

In this study Rotavirus C was found in 4 pigs which were not characteristically diarrheic but from the same household. These however were post weaned of more than 3 months old. Genotyping of products (amplicons) from the first round PCR (Nest 1) amplification yielded only non typeable P and G. The frequency of RVC in this study was 1 (14.3%). RVC was first found in piglets in 1980 (Saif *et al.*, 1980) and has since been acknowledged as a common etiology found in nursing, weaning and post-weaning pigs either in mixed infections with other enteric pathogens or alone (Chang *et al.*, 2012). Adult pigs are reported to have a 100% sero-conversion of the virus.

Steyer *et al.* (2006) reported sporadic gastroenteritis in children and adults in Slovenia while Joshi *et al.* (2017) reported of infection in patients with acute gastroenteritis in outbreaks in western India between 2006 and 2014. Steyer *et al.* (2008) indicated that the risk of transmission of zoonotic porcine or bovine rotaviruses is relatively higher in communities where there's close association between humans and animals exist. Hitherto in Ghana, the few reports on rotavirus C in pigs austencibly have not generated scientific interest as yet, inspite of its occurrence, the sporadic reports of findings of the virus and the highly zoonotic nature of the virus worldwide (Mwenda *et al.* 2014). Seheri *et al.*, (2014) reported that even though sub-Saharan Africa has a high load of RV disease due to high diversity of circulating strains there is still very limited country specific data on RVA genotypes available.

Zoonotic transmission of rotavirus C could not be over emphasised in this situation due to the natural capacity of the pigs to harbour the virus and to transmit to humans in their close proximity. Kim *et al.* (2012) suggested that pigs are being considered as reservoirs for emerging rotavirus infections. Cook *et al.* (2004) and Martella *et al.* (2010) both had previously emphasized the potential zoonotic nature of rotavirus C. Kim *et al.* (2012) describes rotavirus as the major cause of neonatal porcine diarrhoea causing significant losses in the piggery industry. With the increase in animal exploitation in Ghana primarily as a source of subsistence and livelihood, this finding amongst others is important information for disease control in the piggery industry in Ghana.

Limitations in this study were:

- 1) uncooperative nature of some of the subjects in the some of the selected households in Akuse
- 2) The challenge of a readily available and affordable sequencing method to characterize the unusual circulating and untypeable strains found in this study to aid in the determination of the natural reservoirs for these strains.

5.2 CONCLUSION

This study has identified possible reassortant strains of rotaviruses G8 and G10 characteristically of bovine origin in humans from the Akuse district and its environs. The detection of G8 and G10 rotavirus strains in this study in Ghana compliments and proves the existence of these viral genotypes unique to animals in humans (G8 and G10). It also brings to bear the distribution and possible diversity of these strains in the Akuse district and this strengthens the data base for further research of these viruses circulating in

humans in our communities especially in the rural areas where humans and animals live in close association.

The study has also identified rotavirus B in sheep in Ghana, a phenomenon which hitherto has not been reported here yet, thus rendering our result a very important novelty considering the rate of zoonotic disease out breaks caused by the virus in humans and animals alike elsewhere.

It is recommended that:

- 1) Surveillance work in Ghana should not only concentrate on humans but also animals due to increase in findings and reports of the detection of viral strains unique to animals in humans in the country taking into account the zoonotic and public health implications of the disease.
- 2) Reports and findings of zoonotic transmissions of reassortant strains should be considered for viral profiling to allow for a proper viral strain combination selection for vaccines currently being used in the Country's vaccination programmes.
- 3) It is important that the Veterinary Sector in the country incorporates rotavirus disease into their approved OIE list of scheduled diseases for a comprehensive vaccination programme, not only for the control of the disease in animals but also for public health reasons.

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APPENDIXES

Appendix I Chemicals, Reagents and Equipment

1. DNA Extraction and PCR

Chemicals and reagents

1X OneTaq Quick-Load Master Mix with Standard Buffer:

Magnesium Chloride ($MgCl_2$), *Taq* DNA polymerase, Deoxyribonucleotide triphosphates (dATP, dCTP, dGTP and dTTP) [QIAGEN]

Oligonucleotide Primers(Sigma)

100 bp DNA ladder (New England Biolabs Inc., Ipswich, MA, USA).

Agarose (Sigma-Aldrich)

Ethidium Bromide (10mg/ml) [Sigma]

TNES buffer (10Mm Tris, 400Mm NaCl, 100Mm EDTA, 0.6% SDS)

2. Equipment

Microwave oven (LG)

Vortex (Electro Ltd, UK)

Microcomputer Electrophoresis (Power supply, Bioblock Scientific, Consort, Lyon-France. S. N0 022777).

Thermo Block TDB-120, Warren, United States of America)

T3 Thermocycler, Biometra, Biotron, GmBH Germany, S. N0 3406124.

Electrophoresis Tank, Flowgen, UK, S. N0 2110.

UVIDOC HD5 20MX, UVITEC Cambrdge, S. N0 15200552

Heraeus Megafuge 8 Centrifuge, Thermo Scientific, Thermo Electron LED GmbH, Germany. S. N0 721115033109.

WaterBath-Precision, Thermo Scientific, Thermo Scientific, USA. S. N0. 226925-185.

Orbital Shaker, Glass plate set up Hoefler Apparatus SE600, Camera, Bright Light

Appendix II

A: REAGENTS FOR EXTRACTING dsRNA USING BENDER EXTRACTION METHOD

Bender Buffer (100 ml): 0.58 g (0.1) of sodium chloride (NaCl) was added to 6.8 g (2M) sucrose. They were dissolved in 10 ml (0.1 M) of 1M Tris (pH 8.0), 50ml 0.05 of 0.1M EDTA and 2.5 ml (0.5%) of 20% SDS. The volume was made up to 100 ml with distilled water.

Stock Solutions

1M Tris-Hcl (pH 8.0): The stock solution of 1M Tris (100 ml), pH 8.0 was prepared by weighing 12.11 g Tris and the pH adjusted to 8.0 with HCl.

20% SDS: 20 g of SDS dry powder was dissolved in 100 ml DDW.

8M Potassium acetate (KAc): 5 M Potassium acetate (100 ml) was prepared by dissolving 49.1 g KAc in a final volume of 100 ml. 8 M KAc was prepared by taking 60 ml of 5 M KAc and mixing with 11.5 ml glacial acetic. The volume was made up to 100ml with distilled water.

Tris - EDTA (TE) buffer (10 Mm Tris HCl, 1 mM EDTA): 1 ml of stock solution 1 M Tris-HCl (pH 8.0) was taken. 1ml of stock solution 0.1 EDTA (pH 8.0) was added and the volume made up to 100 ml with distilled water.

5 M NaCl (100 ml): 29.22g NaCl was dissolved in a final volume of 100ml distilled water.

B: REAGENTS USED IN THE POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

10% Resolving Gel

	1.5 Gel		0.75 Gel	
	1X	2X	1X	2X
Distilled water	15.8ml	31.6ml	9.9ml	19.8ml
30% Acrylamide Stock	10.0ml	20.0ml	6.3ml	12.5ml
Resolving Buffer (pH 8.9)	3.375ml	7.5ml	2.4ml	4.8ml
TEMED.	15 µl	30 µl	10 µl	1.9 µl
10% Ammonium persulphate	450 µl	900 µl	280 µl	564 µl

30% Stacking Gel

	1.5 Gel		0.75 Gel	
	1X	2X	1X	2X
Distilled water	6.8ml	13.6ml	5.1ml	10.1ml
30% Acrylamide Stock	1.6ml	3.2ml	1.2ml	2.4ml
Spacer Buffer (pH 6.7)	1.25ml	2.5ml	0.9ml	1.9ml
TEMED	5 µl	10 µl	4 µl	8 µl
10% Ammonium persulphate	150 µl	300 µl	112 µl.	225 µl

PAGE Sample dye: 10 mg bromophenol blue was mixed with 5ml spacer gel buffer and 1ml glycerol.

30% Acrylamide stock: 30 g acrylamide and 0.8 g N, N' methylene bis-acrylamide were added to 50 ml distilled water. The solution was stored at 4 in the dark.

1 M Hydrochloric acid (HCl): 86 ml concentrated HCl was added to 910 ml distilled water.

Resolving gel buffer (pH 8.9): 36.4 g of Tris was added to 48 ml 1 M HCl and the pH was adjusted to 8.9 with HCl or sodium hydroxide (NaCl). The volume was made up to 100 ml with distilled water.

Stacking gel buffer (pH 6.7): 5.98 g Tris was dissolved in distilled water and the pH was adjusted to 6.7 with the 1 M HCl. The volume was made up to 100 ml with distilled water.

10% (w/v) Ammonium persulphate (APS): 0.1 g ammonium persulphate was dissolved in 1 ml of distilled water prior to use. The solution was stored at 4°C for maximum of three days.

5X Tris-glycine running buffer: 15.1 g of Tris was added to 94 g of glycine and dissolved. The volume was made up to the 1000ml with distilled water.

1X Tris-glycine running buffer: 200 ml of 5X Tris-glycine buffer was diluted with 800ml of distilled water and used to run the PAGES.

Sample buffer (20ml): 10 ml of stacking gel buffer (pH 6.8) was added to 4 ml glycerol and 20 mg bromophenol blue. The volume was made up to 20 ml with distilled water.

**C: Reagents for VP4 and VP7 Reverse –Transcriptase Polymerase Chain Reaction.
(RT-PCR) (Gouvea at al., 1990, Gentch et al., 1992)**

20X Tris-acetic acid-EDTA (TAE) buffer: 48.44 g of 0.4 M Tris, 6.81 g of 0.05 NaCl and 3.72 g of 0.01 M EDTA were dissolved in distilled water. The pH was adjusted to 7.9 with glacial acetic acid and the volume made up to 1000ml with distilled water.

1X Tris-acetic acid-EDTA buffer (1X TAE): 50 ml of the 20X TAE buffer was added to 950 ml of distilled water.

Ethidium bromide stock: 1 tablet (100 g) of ethidium was dissolved in 10ml of distilled water and the solution stored in the dark or foil-covered foil.

1% Agarose Gel: 0.1 g of agarose (Seaken) was mixed in 100 ml of 1X TAE buffer at pH 7.9. The mixture was heated in a microwave oven until the agarose was completely dissolved. The solution was swirled to cool and 3 µl of ethidium bromide stock was added, mixed and poured into the tray. The comb was inserted and the gel was allowed for at least 30 minutes to set before use.

Appendix III

INFORMATION AND CONSENT FORM

Title:	Characterisation and Genotyping of Rotaviruses from Communities in Akuse District and its Environs
Investigator:	Frederica Lizz Hayford
Institution/Organization	Department of Medical Microbiology, School of Biomedical and Allied Health Sciences

This research aims at carrying out a molecular characterization of rotavirus strains circulating among humans and animals in communities of Akuse where animals and humans live close in proximity. Rotavirus is a major aetiological agent for diarrhoea disease in infants, children and adults alike. Many recent works have proven that interspecies transmission is possible under natural condition, where animals and humans live in close proximity. So, with the increase in the detection of mixed G and P genotypes worldwide, more investigations at genome level is necessary to determine the properties of the mixed infection. This will enable an informed selection of genotypes which can be incorporated into future vaccine formulates for human and animal use for this research, all participants living in close association with animals in their households, both sick and healthy are eligible.

Possible Risk and Discomforts

Procedure for sample collection is devoid of risks, each participant will voluntarily submit to us a moderate pea size amount of their faecal material. Same amount will be taken for their live stock and other animals in their household.

Possible Benefits

Subject's faecal samples will be tested and analysed at no cost to them. Results will inform the need for an appropriate management of their infection, and the possible introduction of rotavirus vaccine for livestock for disease control and prevention.

Confidentiality

All information will be kept confidential. Results of investigation and other information obtained will only be shared with the appropriate authority. Data that may be published will not reveal subject’s identity.

Compensation

There will be no cost or payment to subject for participation in this study.

Freedom to refuse or withdraw

Subject should join the study only if he/she wants to. You can ask questions, you can leave the study at anytime without giving any reason. This will not affect you in anyway.

Contact

This proposal has been reviewed and approved by the University of Ghana, School of Biomedical and Allied Health Sciences and Protocol Review Committee which ensures that participants of the study are protected from harm. If you have any concerns about your rights as a participant, you can contact the Chairman of the Ethical and Protocol Review Committee of the College of Health Sciences, Korle Bu, Accra

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

Date:.....

.....

Signature of researcher

CONSENT FORM

The above document describing the benefits, risks and procedures for the research titled **“Characterisation and Genotyping of Rotaviruses from Communities in Akuse District and its Environs where humans and animals live in close proximity at the Korle Bu Teaching Hospital”** has been read and explained to/caregiver/next of kin. I caregiver have read and understood the above information. I/caregiver have been given opportunity to ask questions about the research and have received satisfactory answers. I/caregiver agree to participate in this study as a volunteer.

Date:.....

Signature/Thumbprint of patient/caregiver

I certify that the nature and purpose, the potential benefits, and possible risks associated.

Date:.....

Signature/Thumbprint of witness

Appendix IV

ROTAVIRUS QUESTIONNAIRE

ROTA VIRUS PROJECT

Date:

Study Number:

House hold information:

1. Location (Neighbourhood, Area, Landmark).....
2. Type of living quarters (Compound house, Self contained walled house; semi-detached). Specify.....
3. How many households (family units) in the living quarters.....
4. How many people in a house hold.....
5. Ages of subject:
Gender: male female
6. How often do you visit the hospital?
7. Disease conditions presented to the hospital.
8. Have you had any case of diarrhoea in the last 3 months?
10. Was it reported to the hospital? Specify: Yes No
11. Do you currently have diarrhoea?
11(a).If yes how long.
Do you keep livestock in the house?
13. What type of animals?
 - i
 - ii
 - iii
 - iv
14. How long have you been keeping animals/livestock?
 - i Less than a year
 - ii A year
 - iii Less than 10 years (Specify).
 - iv More than 10 years.
15. How are the animals kept and fed.
 - i Pens in the house (Intensive)

- ii Partially in pens (Semi intensive)
- iii Free range in the house compound.
- iv Others

16. Who takes care of the animals?

- i) Adults (Age range).
- ii) Children (Age range).
- iii) Others (Specify).

17. Who cleans the pens?

18. How often is it cleaned?

- i) Daily.
- ii) Weekly.
- iii) Bi weekly.
- iv) Others (Specify).....

19. How do you clean the pens/compound.....

- i Sweep only.
- ii Remove bedding only.
- iii Sweep and disinfect.
- iv Others (Specify).

20. How often are the animals routinely treated?

21. Who treats them?

22. Do you go to the Veterinary Clinic?

- i Yes/No.
- ii Self treatment.
- iii Others.

23. What is your source of drinking water?

- i Pipe
- ii Well water.
- iii Others (Specify).

24. How does animal get drinking water?

- i Fetched the water from the well.
- ii Drink from river, lagoon, or poddle.
- iii Others. (Specify).

---Is any of your animals running diarrhoea?

If yes:

---Type of animal? (Specify)

---How long? (Specify)

25. How often do they get diarrhea.

26. Does the feces spread in the compound of the house?

27. Do children come into contact with the fecal material of the animals?

28. Is there any possibility of any fecal material entering your source water?

i) Yes/No

ii) Others (Specify).

29. Is there any particular season of occurrence of the diarrhea.

ii) Yes/No.

30. Is there any particular feed you would attribute the diarrhea to?

The stool: is it any of the following?

i) Watery (does it spread/splash)

ii) Loose

iii) Normal. (Formed heap)

31. Is there blood present?

i) Yes/No

ii) NA....

32. How often do you see blood?

33. Is there mucus. (phlegm)

i) Yes/No

34. How often do you find phlegm?

35. Were they treated, by whom?

i) Self.

ii) Vet.

36. How many died (Young or adults).

37. How did you dispose of the carcass?

38. Were any of the children in the house sick in that period or after?